Mami Slavici, tati Želimiru i sestri Martini.
Dugujem vam sve.

To my mother Slavica, father Želimir and sister Martina.
I owe you everything.

“Nothing is as simple as it seems at first.
Or as hopeless as it seems in the middle.
Or as finished as it seems in the end.”

Found in the Latex PhD template of a witty physics student.

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GHENT UNIVERSITY

Role of sorbic acid in the fungal stability of water-in-oil emulsions

Doctoral thesis

MSc. Irena Šoljić

Thesis submitted in fulfillment of the requirements for the degree of
Doctor of Bioscience Engineering

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For those of you who know me, you know that I like being a member of organizations. It is because these fulfill some of my basic needs, (i) to get to know interesting people and kindred souls, (ii) to achieve goals for the betterment of society. In this aspect three organizations were crucial during my time in Gent and I would like to express my gratitude to them and their members. These are the International Association of Students in Agriculture (IAAS), Global Harmonization Initiative (GHI), and TEDx Ghent. I'd like to emphasize the following people. MSc. Tine Willemyns (IAAS), thank you for telling me about this position. None of this would happen if you didn't encourage me to apply. I will always be in your debt. Dr. h.c. Huub Lelieveld (GHI), thank you for placing your trust in me as General Secretary, for everything that I learned from you and for showing me how healthy and graceful one can grow older. You will always be a role model for me and one of the people that has influenced my life the most. Diána Páti (TEDxGhent), thank you for the most spontaneous Wednesday afternoons one could have in Flanders and for the honest conversations. You are one of the most empathic people I know and I have always felt at home with you. Yonca Braeckman (TEDxGhent), thank you for leading, for saying and doing the uncomfortable and for your impeccable moral compass. You are the most competent woman I have met in Gent. All of you influenced everything that I am to become and helped eased the solitude of my doctoral work.

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P.S. To the reader, thank you for reading! You are probably a friend/acquaintance or a PhD or Master student in need of information. I want to tell you that you will fail and you will be lost, and it will be fine. Stick to it. The completion of a long and difficult task is one of the biggest joys of one's life. And remember what Les Brown says – It's not over until you win!

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TABLE OF CONTENTS

LIST OF SYMBOLS

$\mathbf{((HA)_{aq,eq}+(A^-)_{aq,eq})}$	Total aqueous sorbic acid (mg/kg)
$\mathbf{(A^-)_{aq,eq}}$	Conjugate base of sorbic acid at equilibrium (mg/kg)
$\mathbf{(HA)_{aq,eq}}$	Undissociated (inhibitory) aqueous sorbic acid at equilibrium (mg/kg)
$\mathbf{(HA)_{aq,i}}$	Initial sorbic acid in the aqueous phase (mg/L)
$\mathbf{(HA)_{lip,eq}}$	Undissociated sorbic acid in lipid phase at equilibrium (mg/kg)
$\mathbf{(HA)_{tot,eq}}$	Total sorbic acid added in system (mg/kg)
$\boldsymbol{MW_{HA}}$	Molecular weight of sorbic acid (g/mol)
$\boldsymbol{m_{HA_{oil,i}}}$	Mass of sorbic acid added to the oil phase (g)
$\boldsymbol{n_{HA_{oil,i}}}$	Initial moles of sorbic acid added to oil phase (mol)
$\boldsymbol{n_{HA_{tot}}}$	Total moles of sorbic acid present in the system (mol)
$\boldsymbol{\rho_{aq}}$	Volumetric mass (density) of the aqueous phase (kg/L)
$\boldsymbol{\rho_{lip}}$	Volumetric mass (density) of the lipid phase (kg/L)
$\mathbf{[A^-]_{aq,eq}}$	Conjugate base of sorbic acid at equilibrium (mol/L)
$\mathbf{[A^-]_{oil,eq}}$	Conjugate base of sorbic acid in oil phase (mol/L)
$\mathbf{[HA]_{aq,eq}}$	Undissociated (inhibitory) aqueous sorbic acid at equilibrium (mol/L)
$\mathbf{[HA]_{lip,eq}}$	Undissociated sorbic acid in lipid phase at equilibrium (mol/L)
$\mathbf{[HA]_{oil,eq}}$	Undissociated sorbic acid in oil phase at equilibrium (mol/L)
$\mathbf{[HA]_{oil,eq}}$	Undissociated sorbic acid in oil phase at equilibrium (mol/L)
$\mathbf{[HA]_{oil,i}}$	Initial concentration of sorbic acid in oil phase (mol/L)
$\mathbf{[HA]_{tot}}$	Total sorbic acid added in system (mol/L)
$\boldsymbol{\mu_{max}}$	Maximum specific growth rate
A(t)	Gradual delay in time
$\mathbf{D_{43}}$	Volume weighted mean diameter (µm)
$\mathbf{e^{\sigma}}$	Geometric standard deviation of the droplet size distribution
$\mathbf{f_{lip}}$	Solid fat content expressed on lipid fraction of W|O emulsion (%)
$\mathbf{f_{tot}}$	Solid fat content expressed on total W|O emulsion (%)
$\mathbf{K_a}$	Acid dissociation constant
$\mathbf{K_d}$	Distribution coefficient (apparent partition coefficient)
$\mathbf{K_p}$	Partition coefficient
m	Curvature parameter
$\mathbf{m_{aq}}$	Mass of aqueous phase (kg)
$\mathbf{m_{lip}}$	Mass of lipid phase (kg)
$\mathbf{m_{tot}}$	Total mass of W|O emulsion (kg)
$\mathbf{MW_{H+}}$	Molecular weight of a hydrogen ion (g/mol)
$\mathbf{pK_a}$	Log acid dissociation constant
r	Mass fraction of lipid phase in W|O emulsion (-)
r(m)	Radius of the longest axis of a contaminating microorganism
$\mathbf{V_{aq}}$	Aqueous phase volume (L)
$\mathbf{V_{oil}}$	Oil phase volume (L)
$\mathbf{V_{tot}(r)}$	Total volume of droplets with radius r
$\mathbf{y_{max}}$	Natural logarithm of the maximum cell concentration
$\mathbf{y_o}$	Natural logarithm of the cell concentration at $t=t_0$
$\boldsymbol{\alpha_0}$	Physiological state of cells at $t=t_0$
λ	Lag phase duration
$\boldsymbol{\varphi}$	Volume fraction of oil in the system (-)

LIST OF ABBREVIATIONS

AF	Accuracy factor
AMF	Anhydrous milk fat
BF	Bias factor
BPW	Buffered peptone water
CFU	Colony forming units
CLSM	Confocal laser scanning microscopy
DSD	Droplet size distribution
DSD-I	Droplet size distribution influence
GFP	Green fluorescent protein
GT	Generation time
GT	Generation time
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatography
MEA	Malt extract agar
MIC	Minimum inhibitory concentration
NMR	Nuclear magnetic resonance
O|W	Oil-in-water emulsion
O|W|O	Oil-in-water-in oil emulsion
OD	Optical density
PBS	Phosphate buffered saline
pfg-NMR	Pulsed field gradient nuclear magnetic resonance
PGPR	Polyglycerol polyricinoleate
PPS	Peptone hysiological Salt
SFC	Solid fat content
W|O	Water-in-oil emulsion
W|O+E	Model water-in-oil emulsion with emulsifier
W|O+F	Model water-in-oil emulsion with solid fat
W|O|W	Water-in-oil-in-water emulsion
YEG	Yeast extract glucose media
YES	Yeast extract sucrose semi-solid media
YNA	Yeast nitrogen agar
YNB	Yeast nitrogen broth
YNB+SA	Yeast nitrogen broth with sorbic acid
YNB+SA|oil	Yeast nitrogen broth with sorbic acid and oil on top
YNB+SA|oil+fat	Yeast nitrogen broth with sorbic acid and an oil and fat mixture on top

AIM OF THE STUDY

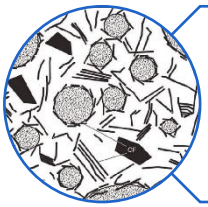
Confidence in the safety and quality of food is an important requirement for consumers. The ultimate responsibility for ensuring safe and wholesome food lies with food producers. During the past decades, important advances in the field of food preservation have paved the way towards ensuring food safety and quality to a greater extent. Water-in-oil (W|O) emulsions such as margarine, butter and fat spreads have become staple foods in households worldwide and are also extensively used in the bakery and confectionary industry. Most of these products are vulnerable to fungal spoilage, especially when absence of preservatives and/or low fat contents is required. The main factors that determine the fungal stability of W|O emulsions are indeed the water content and availability of nutrients, water droplet size distribution (DSD) and the presence of preservatives, such as sorbic acid.

The goal of this study was to investigate the role of sorbic acid on the fungal stability of W|O emulsions. First, a theoretical model was developed that describes the distribution of sorbic acid in W|O emulsions, taking into account the pH, mass ratio of lipid phase and solid fat content (SFC). These assumptions were then experimentally validated. As W|O emulsions are complex biphasic systems with many components in both the water and lipid phase, it was decided to study the phenomena by simplifying the food matrix into its principal components before real food validation. The purpose of the sorbic acid distribution model was to predict undissociated aqueous sorbic acid concentrations. It is the undissociated sorbic acid form that is predominantly responsible for antifungal action, however it also preferentially partitions into the lipid phase. This is considered as a loss of the preservative. Initial hypotheses about sorbic acid behavior in W|O emulsions were previously mentioned in literature, but these statements regarded the lipid phase as a homogenous mass. In this work it was assumed that the lipid phase of W|O is not a homogenous mass because it consists of a network of liquid oil and solid fat. The presence of solid fat was then taken into account in the theoretical model as it was assumed that it influences sorbic acid distribution and consequentially its preservative effect on fungi in the aqueous phase. Emulsion making is a complex process, involving the process of emulsification i.e. combining the aqueous and lipid phase into a stabile matrix by using emulsifiers that increase the kinetic stability of the system. This is achieved by adding emulsifiers which aid in reducing interfacial tension between the two immiscible phases. However, no information is available on the effect of emulsifiers on the distribution of sorbic acid. Therefore, the influence of emulsification on sorbic acid distribution in W|O emulsions was also investigated.

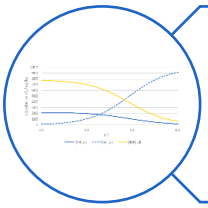
Recently, market demand is shifting towards products with less additives. Thus, it becomes increasingly important to investigate other intrinsic and extrinsic factors contributing to microbial stability of foods. In this work the effect of two important factors, more specifically droplet size distribution (DSD) and temperature, were investigated on their influence on fungal growth in W│O emulsions.

Lastly, in order to test the assumptions provided by the theoretical model and experimental data from the model W│O emulsions, industrial validation on real food products was performed. First, emulsion spoiling molds were inoculated in semi-solid media with sorbic acid to determine minimum inhibitory concentrations (MIC) inhibiting the growth of the molds. Sorbic acid concentrations equivalent to those elucidated as MICs in semi-solid media were applied in the industrially produced W│O emulsions. The applied sorbic acid concentrations were calculated over the proposed sorbic acid distribution model, taking into account the final pH, mass ratio of lipid phase and measured solid fat content (SFC) of the W│O emulsion. The real-food validation of the proposed model is meant to serve as proof for the industry that information from this study might further the efforts of designing microbiologically stabile W│O emulsions and reduce the occurrence of fungal spoilage incidents.

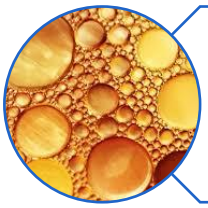
OVERVIEW OF THE THESIS



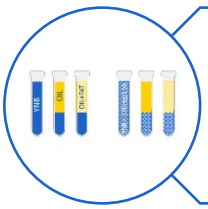
Chapter 1: Introduction



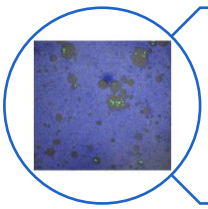
Chapter 2: Effect of pH, mass ratio of lipid phase and solid fat on sorbic acid distribution in model W|O and W|O+F systems



Chapter 3: Effect of emulsification on sorbic acid distribution in model W|O+E systems

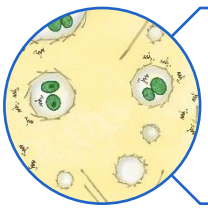


Chapter 4: Solid fat influences sorbic acid partitioning and enhances the preservation effect on *C. guilliermondii* in biphasic food model systems



Chapter 5: Influence of temperature and emulsion water droplet size distribution (DSD) on growth of *C. guilliermondii* in recombined butter

Chapter 6: Sensitivity of various emulsion spoiling molds towards sorbic acid and validation of sorbic acid distribution model in industrially produced fat spreads



General conclusion and perspectives

SUMMARY

The present thesis investigates the factors influencing the role of sorbic acid in fungal stability of water-in-oil (W│O) emulsions.

In **Chapter 1**, an overview of the literature regarding the characteristics and microbiological spoilage of W│O emulsions and existing knowledge about sorbic acid behavior in such matrices is given.

In **Chapter 2**, a theoretical model quantifying aqueous sorbic acid concentrations in W|O emulsions was developed in Microsoft® Excel®, taking into account pH, mass fraction of lipid phase and solid fat content (SFC). The predictions were validated by making model W|O and W|O+F (water|oil+fat) systems, comprising of phosphate buffers, sunflower oil and palm stearin, which represented the major constituents of W│O emulsions. Total sorbic acid in the aqueous phase after equilibrium, $((HA)_{aq,eq}+(A^-)_{aq,eq})$, was quantified by high performance liquid chromatography (HPLC) analysis. The measured values were in very good accordance with the expected values in model W|O systems. In model W|O+F systems, the deviations between the measured and expected data were slightly higher than in model W|O systems. $((HA)_{aq,eq}+(A^-)_{aq,eq})$ concentrations measured in model W|O+F systems were generally higher than those expected, especially in samples with a large SFC. This implies that the presence of solid fat in W│O emulsions hinders sorbic acid partitioning.

Chapter 3 describes the influence of emulsification on the distribution of sorbic acid in model W|O and W|O+E (water|oil+emulsifier) systems, made as a simple representation of emulsified W│O emulsions. Polyglycerol polyricinoleate (PGPR) was used as the emulsifier and the model systems were prepared by emulsification with Ultra Turrax. Slightly more sorbic acid was retrieved in the lipid phase of the centrifuged W|O+E system than in the lipid phase of the W|O system subjected to the same conditions. Nevertheless, due to the narrow experimental setup of the study it is difficult to draw a definite conclusion about the impact of emulsification on sorbic acid distribution in emulsions.

In **Chapter 4**, the influence of structure on sorbic acid distribution and its preservation effect on *Candida guilliermondii* was investigated in model W│O systems comprising of Yeast Nitrogen Broth (YNB) buffered at different pH's, sunflower oil and/or palm stearin. Growth parameters, generation time (GT) and lag phase (λ) of *C. guilliermondii* in the aqueous phase of the food model systems were quantified during 1 month at 7°C and maximum 80 hours at 22°C. HPLC analyses were performed to evaluate $((HA)_{aq,eq}+(A^-)_{aq,eq})$ in each food model

system. Results showed that liquid oil and solid fat have an effect on the aqueous sorbic acid concentration and its preservation effect. The presence of solid fat reduced the tendency of sorbic acid to partition into the lipid phase (as observed in Chapter 2), which enhanced the inhibitory effect of sorbic acid on *C. guilliermondii.*

Chapter 5 describes the results of investigating the influence of temperature (7 and 22 °C) and emulsion water droplet size distribution (DSD), on the growth of *C. guilliermondii* in recombined butter made with anhydrous milk fat (AMF) (61 and 82%) during 21 days of storage. Prior to this, different DSDs were achieved by varying Ultra Turrax rotation speed (5000-15000 rpm) during mulsification. The proportion of microbiologically vulnerable water droplets (diameter > 10 µm) increased as Ultra Turrax rotation speed during emulsification decreased. Also, stabile (diameter < 10 µm) water droplets were more likely to occur in 82% AMF recombined butter than in 61% AMF butter, irrespective of rotation speed during emulsification. Refrigeration for 21 days at 7 °C contributed to keeping the proportion of microbiologically vulnerable water droplets stabile, in both 82 and 61% AMF recombined butter. At 22 °C, the proportion of vulnerable droplets in both 82 and 61% AMF recombined butter increased during 21 days of storage.

An important contribution of this study to the existing knowledge about fungal stability of W│O emulsions was the experimental validation of the proposed model on industrially produced W│O emulsions inoculated with various emulsion spoiling molds. In **Chapter 6**, the sensitivity of these molds towards sorbic acid was investigated, first by elucidating minimum inhibitory concentrations (MICs) in semi-solid microbiological culture media and then validating these findings in industrially produced W│O emulsions.The $(HA)_{tot,eq}$ to be added into W│O emulsions that is necessary to obtain the $(HA)_{aq,eq}$ elucidated as inhibitory in semi-solid media experiments, was calculated over the proposed model (**Chapter 2**). Generally, less $(HA)_{aq,eq}$ was necessary to inhibit mold growth in W│O emulsions than in YEG semi-solid media. This finding emphasizes the importance of validation of microbiological preservative sensitivity studies in culture media on real food matrices.

In the general conclusion and perspectives, the implications of the findings from experimental chapters are given, as well as possible future applications. Also, several other factors governing sorbic acid behavior and fungal stability in W│O emulsions are discussed, to guide further research in this field. Although there is still research to be done regarding the fungal stability of W│O emulsions, this study is an important step forward in helping to formulate quality products with a long shelf-life.

SAMENVATTING

In **hoofdstuk 1** wordt een overzicht gegeven van de literatuur over karakterisatie en microbieel bederf van W│O emulsies en de bestaande kennis over het gedrag van sorbinezuur in deze matrix wordt weergeven.

In **hoofdstuk 2** werd een theoretische model dat de waterige sorbinezuur concentraties in W│O emulsies kwantificeert besproken. Dit model werd ontwikkeld in Microsoft® Excel® en houdt rekening met de pH, de massa fractie van de vet fase en de hoeveelheid vast vet (SFC). De voorspellingen werden gevalideerd door een model W|O en W|O+F (water-in-olie+vet) systeem dat rekening hield met de de belangrijkste componenten van de W|O emulsies: fosfaat buffers, zonnebloem olie en palm stearine. De totale hoeveelheid sorbinezuur in de waterige fase na equilibrium ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) werd gekwantificeerd door middel van high performance liquid chromatography (HPLC) analyse. De gemeten waarden kwamen overeen met de voorspelde waarden uit het model voor W|O emulsies. In het W|O+F model waren de afwijkingen tussen de gemeten en voorspelde waarden iets hoger dan in het W|O model. De gemeten ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) concentraties in het W|O+F model waren hoger dan de voorspelde waarden, voornamelijk in stalen met hoge SFC. Dit impliceert dat de aanwezigheid van vast vet in W|O emulsies de partitionering van sorbinezuur hindert.

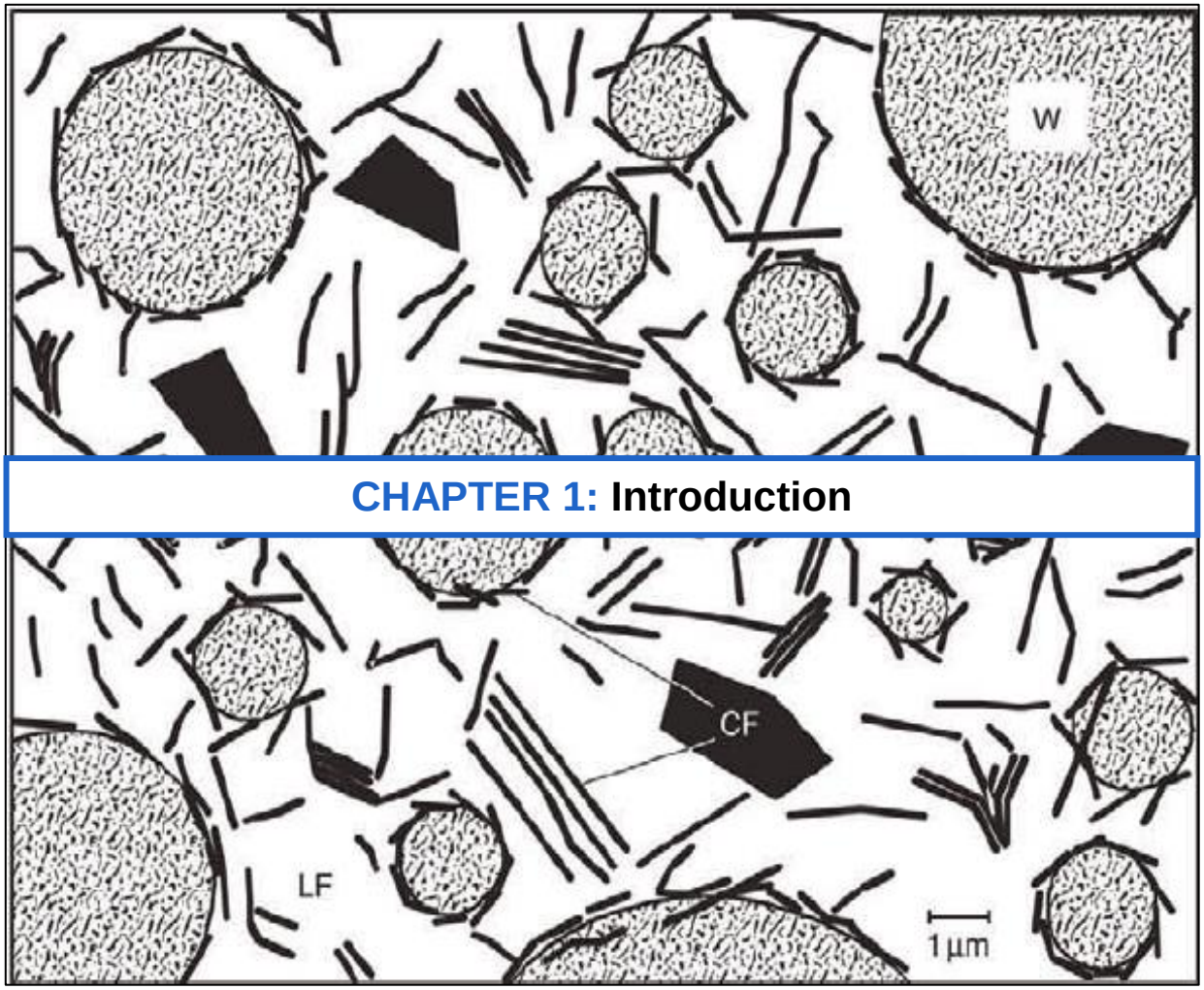
Hoofdstuk 3 beschrijf de invloed van emulsificatie op de distributie van sorbinezuur in een W|O model en W|O+E (water-in-olie+emulgator) systemen, die gebruikt worden als een eenvoudige voorstelling van geëmulgeerde W|O emulsies. Polyglycerol polyricinoleate (PGPR) werd gebruikt als emulgator en het model system werd bereid door emulsificatie met de Ultra Turrax. Er werd iets meer sorbinezuur uit de vet fase bekomen door de centrifugatie van de W|O+E systemen, dan in de vet fase van de W|O systemen bij dezelfde condities. Het is echter moeilijk om een beslissende conclusie te trekken over de impact van de emulsificatie op sorbinezuur verdeling in emulsies door de beperkte experimentele setup van de studie.

In **Hoofdstuk 4** werd de invloed van de structuur op zowel de sorbinezuur distributie als het conserverend effect op *Candida guilliermondii* onderzocht in W│O emulsies bestaand uit Yeast Nitrogen Broth (YNB) gebufferd op verschillende pH's, zonnebloemolie en/of palm stearine. Groeiparameters, generatietijd (GT) en lag-fase (λ) van *C. guilliermondii* in de waterige fase van de voedingsmodelsystemen werden opgevolgd gedurende 1 maand bij 7°C en maximum 80 uur bij 22°C. HPLC analyses werden uitgevoerd om de ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) te evalueren in elk voedingsmodelsysteem. De resultaten tonen dat vloeibare olie en vast vet een effect hadden op de waterige sorbinezuurconcentratie en het conserverend effect. De aanwezigheid

van vast vet verminderde de neiging van sorbinezuur om te partitioneren naar de vet fase (zoals gezien werd in hoofdstuk 2) en dit versterkte het inhiberend effect van sorbinezuur op *C. guilliermondii*.

Hoofdstuk 5 beschrijft de resultaten van onderzoek naar de invloed van temperatuur (7 en 22°C) en waterdruppelgrootteverdeling van de emulsie op de groei van *C. guilliermondii* in gerecombineerde boter gemaakt met vochtvrij melkvet (61 en 82%) gedurende 21 dagen bewaring. Hiervoor werden verschillende waterdruppelgrootteverdelingen bekomen door het variëren van de rotatiesnelheid van een Ultra Turrax (5000-15000 rpm) tijdens emulsificatie. Het aandeel microbiologisch kwetsbare waterdruppels (diameter > 10µm) steeg naargelang de rotatiesnelheid van de Ultra Turrax tijdens emulsificatie daalde. Daarnaast waren stabiele waterdruppels (diameter <10µm) meer waarschijnlijk in gerecombineerde boter met 82% vochtvrij melkvet dan gerecombineerde boter met 61% vochtvrij melkvet, onafhankelijk van de rotatiesnelheid tijdens emulsificatie. Gekoelde bewaring voor 21 dagen bij 7°C droeg bij tot het stabiel houden van de proportie microbiologisch stabiele waterdruppels, zowel in gerecombineerde boter met 82% vochtvrij melkvet als in gerecombineerde boter met 61% vochtvrij melkvet. Bij 22°C steeg bij beide boters de proportie kwetsbare druppels gedurende 21 dagen opslag. Een belangrijke bijdrage van deze studie tot de bestaande kennis inzake fungale stabiliteit van W|O emulsies was de experimentele validatie van het voorgestelde model voor W|O emulsies geïnoculeerd met diverse bederfschimmels typisch voor emulsies. In **Hoofdstuk 6** werd de gevoeligheid van deze schimmels voor sorbinezuur bestudeerd. Daartoe werden eerst minimale inhiberende concentraties in halfvaste microbiologische cultuurmedia opgehelderd, om vervolgens de bevindingen te valideren in industrieel geproduceerde W|O emulsies. De $(HA)_{tot,eq}$ die diende toegevoegd te worden in W|O emulsies om de $(HA)_{tot,eq}$ te bekomen die als inhiberend werd bevonden in de halfvaste media-experimenten werd berekend met het voorgestelde model (**Hoofdstuk 2**). Algemeen gezien was minder $(HA)_{tot,eq}$ nodig om schimmelgroei in W|O emulsies te voorkomen dan in halfvaste YEG media. Deze bevinding benadrukt het belang van validatie op reële voedingsmatrices van microbiologische bewaargevoeligheidsstudies die uitgevoerd werden op cultuurmedia.

In de algemene conclusie en perspectieven worden de implicaties van de bevindingen uit de experimentele hoofdstukken gegeven, net als mogelijke toekomstige toepassingen. Daarnaast worden verschillende andere factoren inzake het gedrag van sorbinezuur en fungale stabiliteit in W|O emulsies besproken, om verder onderzoek in dit domein in goede banen te leiden. Hoewel er nog verder onderzoek dient uitgevoerd worden naar de fungale stabiliteit van W|O emulsies, zet dit onderzoek toch een belangrijke stap voorwaarts om te helpen bij het formuleren van kwaliteitsvolle producten met een lange bewaarperiode.



CHAPTER 1: Introduction

INTRODUCTION

Emulsion science is a multidisciplinary subject that combines chemistry, physics and engineering (Evans and Wennerstrom, 1994). The goal of an emulsion scientist working in the food industry is to utilize the principles and techniques of emulsion science to enhance the quality of food and efficiency of food production (McClements, 2015).

Food emulsions, such as mayonnaise, butter and salad dressings are two-phase systems of immiscible liquids with limited stability (Yang and Lai, 2003). One of the phases exists as discrete droplets suspended in the other, continuous phase and there is an interfacial layer between the two phases which is occupied by a necessary surfactant material. Two types of emulsion are important in foods. In oil-in-water (O|W) emulsions, droplets of oil are suspended in a water-continuous phase. These are more versatile emulsions; they appear in food in many forms such as mayonnaises, cream liqueurs, creamers, whippable toppings and ice cream mixes. Examples of water-in-oil (W|O) emulsions are butter, margarines and fat spreads (Dalgleish, 2006). Figure 1.1. shows a schematic representation of an O|W and a W|O emulsion.

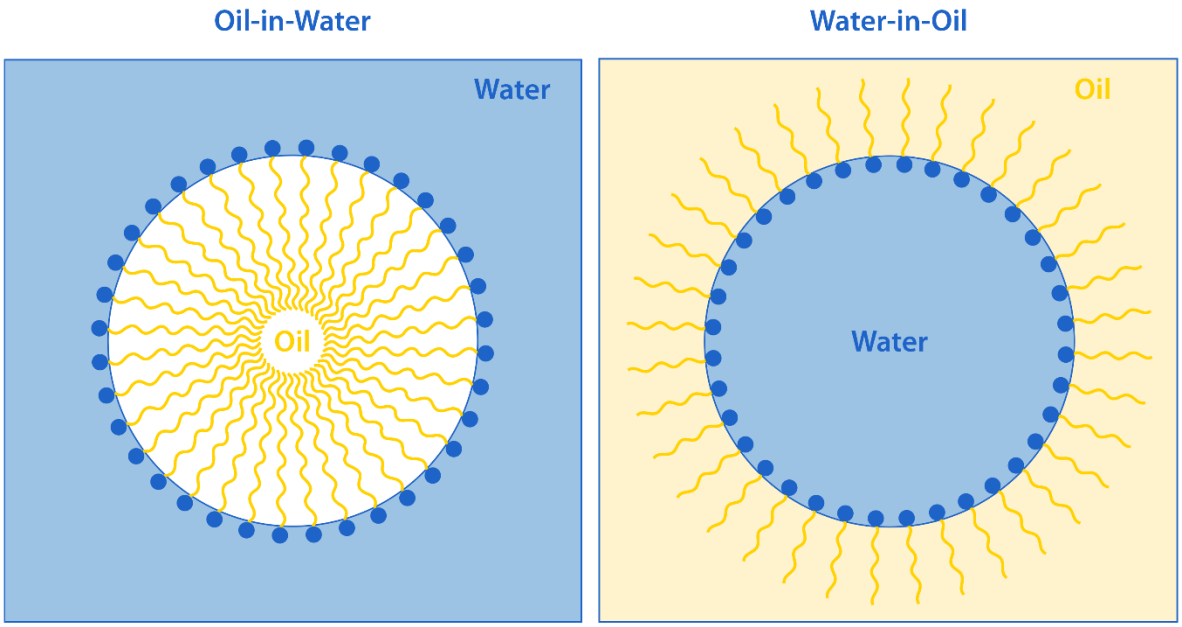


Figure 1.1. Schematic representation of an oil and water droplet in O|W and W|O emulsions stabilized by emulsifiers. Hydrophobic tails of the emulsifier molecules orient themselves towards the oil phase while hydrophilic heads orient themselves towards the water phase.

It is also possible to prepare multiple emulsions such as oil-in-water-in-oil (O|W|O) or water-in-oil-in-water (W|O|W) type emulsions. Research has been carried out to create stabile multiple emulsions which can be used to control the release of certain ingredients, reduce the total fat content of emulsion-based food products or isolate one ingredient from another

(Dickinson and McClements, 1995). Emulsion-based food products exhibit a wide variety of different physicochemical and organoleptic characteristics, such as in appearance, aroma, texture, taste and shelf life. This diversity is the result of the different sorts of ingredients and processing conditions used to create each type of product (McClements, 2015). Presently, W|O emulsions exist in the market with fat levels ranging from 20 to 90%, whereas products with fat levels as low as 3% have been successfully introduced in the USA, UK and The Netherlands (van Zijl and Klapwijk, 2000). Spreads made exclusively from milk fat are commonly referred to as butter or dairy spreads. Spreads made with vegetable or animal fat are called margarines or fat spreads. Spreads made from vegetable and animal fats, with a milk fat content of 10 to 80% of the total fat content, are called blends (EC, 1994). A schematized classification of sales descriptions of spreadable fats according to EC EU 2991/94 is given in Table 1.1.

Table 1.1. EC Sales descriptions of "Spreadable fats". (Adapted from EC EU 2991/94 and Chandan et al., 2015)

	Sales descriptions		
Fat content ranges	**Milk fat product**	**Vegetable/animal fat products**	**Mixed milk fat and vegetable/animal fat**
80% ≤ X < 90%	Butter	Margarine	Blend
62% < X < 80%	Dairy spread X%	Fat spread X%	Blended spread X%
60% ≤ X ≤ 62%	Three-quarter fat butter Reduced fat butter	Three-quarter fat margarine Reduced fat margarine	Three-quarter fat blend Reduced fat blend
41% < X < 60%	Dairy spread X% Reduced fat dairy spread X%	Fat spread X% Reduced fat spread X%	Blended spread X% Reduced fat blended spread X%
39% ≤ X ≤ 41%	Half-fat butter Low-fat butter Light butter	Half-fat margarine Low-fat margarine Light margarine	Half-fat blend Low-fat blend Light blend
X < 39%	Dairy spread X% Low-fat dairy spread X% Light dairy spread X%	Fat spread X% Low-fat spread X% Light fat spread X%	Blended spread X% Low-fat blend X% Light blend X%

1.1. Production of food W|O emulsions

The manufacture of a W|O emulsion-based food product with certain quality attributes depends on the selection of the most appropriate raw materials (e.g. water, oil, emulsifiers, thickening agents, vitamins, flavors, colorants, etc.) and processing conditions (e.g. mixing, homogenization, pasteurization, crystallization, etc.) (McClements, 2015).

Prior to converting separate lipid and water phases into an emulsion, it is necessary to disperse the various ingredients into the phase in which they are most soluble. Oil-soluble ingredients, such as low hydrophilic-lipophilic balance (HLB) value emulsifiers, vitamins, colors and

antioxidants, are usually mixed with the lipid phase (McClements, 2015) and kept at 45-60 °C before emulsification (ICMSF, 2005). A wide range of oils and fats may be used in the formulation, e.g. soya, palm, sunflower and fish oil. The type of fat used depends on economic constraints, such as availability and cost, but also on the required functional specifications with regard to emulsion stability and shelf life, cooking performance, spreadability at refrigerator temperature, flavor release and appearance (Madsen, 1989, 1990; Moustafa, 1990). If the lipid phase contains any crystalline matter, it is necessary to warm it to a temperature where all the fat melts prior to homogenization (Mulder and Walstra, 1974; Phipps, 1985).

The interfacial region may contain a mixture of various surface-active components, including proteins, emulsifiers and solid particles. These components may form various types of structural entities in the oil, water or interfacial regions, such as fat crystals, protein aggregates, air bubbles and emulsifier micelles (McClements, 2015).

The aqueous phase contains all water-soluble ingredients such as proteins, polysaccharides, sugars, salts, vitamins, colors, antioxidants and high HLB value emulsifiers. The pH of the water phase of a W|O emulsion is usually set between pH 3.5 and 6.5 (ICMSF, 2005; McClements, 2015). Salt or brine is added to fat spreads primarily to improve taste and prevent growth of microorganisms. In a margarine with 16% water, addition of 1% salt overall will inhibit the growth of many microorganisms whereas addition of 2% will inhibit almost all. 1% of salt overall in a 16% water margarine corresponds to 6% salt in the water phase (Young and Wassell, 2008). Milk proteins such as skim milk and whey powder have an O|W emulsifying effect, which work against the W|O emulsifier system and thus destabilize the W|O emulsion. However, the presence of milk proteins and their working against the W|O emulsion, enhances flavor release (Kirkeby, 2006).

Basic W|O emulsion processing comprises of five operations – emulsification, cooling (supercooling), working (plastication), resting and packaging. Emulsification is a process of converting two immiscible liquids into an emulsion (also known as homogenization) and the device designed to carry out this process is called a homogenizer. The intensity and duration of the mixing process depend on the time required to solubilize and uniformly distribute the phases (McClements, 2015). In the case of W|O emulsions, the resultant pre-emulsion is a stabile fat-continuous mixture throughout which the water phase is dispersed. A high-pressure pump feeds this mixture through a closed system of scraped surface heat exchangers and mixers, which cool it down to 10-20 °C and work it into a fine emulsion. The product is then packed into tubs (soft spreadable products) or in wrappers (hard margarines used for baking

and cooking) and allowed to rest and crystallize completely on refrigeration temperature (ICMSF, 2005).

1.2. Chemical composition of food W|O emulsions

Butter, margarine and fat spreads are W|O emulsions that usually remain solid at room temperature. Fat, emulsifier, milk protein and water affect the emulsion and processing characteristics of the final product. Other ingredients, such as preservatives, colors and flavors, do not affect product processability (Belitz et al., 2009). Other than milk fat and water, butter contains 0.5– 4.0% fat-free solids and 1.2% NaCl in the case of salted butter. Margarine contains 80 to 90% fat and the rest is the emulsified water phase. It is usually stabilized by a mixture of mono- and diacylglycerols (approx. 0.5%) and crude lecithin (approx. 0.25%) (Keogh, 2006).

The lipid phase of food W|O emulsions is partly crystalline and it may be subject to chemical change such as lipolysis or oxidation (Dalgleish, 2006). Fats of animal and vegetable origin contain about 99% triglycerides (Keogh, 2006). The functional behavior of a fat or blend of fats will depend on (i) the melting point of the fat, (ii) the content of solid or crystalline triglycerides present at any temperature but especially in the range of 4–37 °C, (iii) the form of the crystals present (Mostafa et al., 1985).

The pH of the water phase of fat spreads is usually set with the addition of citric and lactic acids. This not only affects the flavor, but protects against microbial growth and influences as such microbial safety as well as spoilage. Margarine can contain aroma substances. Readily available compounds, such as diacetyl, butyric acid, lactones of C8–C14 hydroxy-fatty acids and (Z)-4-heptenal, may be used for aromatization. Margarine can be colored with β-carotene or with gently refined, unbleached palm oil. Some products are vitaminized by the addition of about 25 IU/g vitamin A and 1 IU/g vitamin D2 (Belitz et al., 2009).

1.2.1. Preservatives in food W|O emulsions

Many foods rely for their preservation on the addition of organic acids in the system. Acetic, benzoic, lactic and sorbic acid (and their salts) are added as antimicrobials in foods. Acetic and lactic acid are generally considered as acidulants that exhibit an inhibitory effect when high concentrations are applied, while sorbic and benzoic acid are weak organic acids that are considered as true preservatives, effective even in small concentrations (Devlieghere et al., 2013). Their preservative action is a consequence of the combination of the pH of food and

antimicrobial properties of the undissociated form of the molecule. The antimicrobial effect is influenced by the fundamental thermodynamic characteristics of dissociation and partition. In addition, the concentration of sugars or salts in a food product can contribute to the preservation effect by decreasing the water activity (Brocklehurst, 2003).

Weak organic acids dissociate depending on the pH of the environment. This dissociation is key to prediction of the concentration of the undissociated form of the acid, which has the predominant antimicrobial effect in foods (Baird-Parker, 1980; Eklund, 1983). The sensitivity of microorganisms to weak organic acids has been shown to be a significant species and strain-dependent parameter (Eklund, 1985). The addition of a weak acid to a food may lead to the preferential growth of species tolerant to weak acids (Baird-Parker, 1980).

EC EU 1333/2008 allows adding sorbic acid and its salts as preservatives in fat spreads; permissible sorbic acid concentrations are 1000 mg/kg (0.1%) in W|O emulsions with more than 60% fat and 2000 mg/kg (0.2%) in W|O emulsions with less than 60% fat. To ensure a sufficient level of the undissociated acid, the pH should be sufficiently low, preferably below the pKa, 4.75. The behavior of sorbic acid in biphasic systems like fat spreads is further elucidated in Paragraph 1.5.

1.2.2. Emulsifiers in food emulsions

Emulsifiers are surface-active molecules which adsorb to the surface of formed droplets. Most emulsifiers are amphiphilic molecules (i.e. they have polar and nonpolar regions on the same molecule). The most common emulsifiers used in the food industry are amphiphilic proteins and small-molecule surfactants (McClements, 2015).

Small-molecule surfactants (glycerol esters of fatty acids, phospholipids, etc.), contain long-chain fatty acid residues, which provide (i) the hydrophobic group that binds to the lipid phase and (ii) the hydrophilic group such as glycerol (in monoglycerides and diglycerides) or substituted phosphoglyceryl moieties (in phospholipids) that binds to the water phase of the W|O interface. Such molecules can have an HLB value from 0 (oil soluble) to 20 (water soluble) (McClements, 2015). The HLB value is a measure of the amphiphilic character of emulsifiers (Genot et al., 2013). As a general rule, emulsifiers of a low and high HLB are used to form W|O emulsions and O|W emulsions, respectively.

Polyglycerol polyricinoleate (PGPR, E 476), a polyglycerol ester of castor oil fatty acids, is used as an emulsifier in tin-greasing emulsions for the baking trade, as a viscosity modifier in the

chocolate industry or for the production of low-fat spreads (Bastida-Rodríguez, 2013). EU regulation 1333/2008 allows adding up to 4000 mg/kg PGPR to spreadable fats as defined in Article 115 of and Annex XV to Regulation (EC) No 1234/2007, having a fat content of 41% or less.

1.3. Microbiology of food W | O emulsions

The fat-continuous nature of W | O emulsions has a strong impact on their microbiological stability. The inability of microorganisms to move between water droplets is an important intrinsic preservation factor. Fat can act as a barrier to microbial growth and for this reason fat-continuous systems are usually more stabile than water-continuous systems (ICMSF, 2005). Fats and oils can be metabolized by various fat-metabolizing microorganisms if the conditions for growth are favorable, e.g. temperature, moisture or availability of low-molecular weight nutrients. Enzymes produced by contaminating lipolytic flora can hydrolyze the fat into free fatty acids and trigger fatty acid oxidation (ICMSF, 2005). At the same time, fats and oils can protect microorganisms so that they may survive heat inactivation for quite some time (Troller and Christian, 1978; Hersom and Hulland, 1980; Gaze, 1985). This would present a hazard if the microorganisms were pathogens. A protective effect by oils and fats during heat inactivation is only found in the absence of water or in the presence of very low levels. Margarine has a large amount of water (10-20% on product mass) and it is not expected that there would be a large protective effect of the oil towards microorganisms (Sagdic et al., 2017).

Closed shelf-life of fat spreads is generally limited to 3-6 months, often for non-microbiological reasons, such as lipid oxidation. More microbiologically sensitive product formulations may require refrigerated storage, especially during consumer use. At that moment, the product may become contaminated with a variety of microorganisms originating from the air, bread crumbs or from other foods. With very vulnerable products, e.g. non-preserved low-fat spreads containing a dairy ingredient or water-continuous spreads, the shelf-life duration may be quite limited due to microbiological deterioration (ICMSF, 2005). The microbiological stability of fat spreads is influenced by product composition (i.e. correct pH, the appropriate level of ingredients and fine water dispersion) (Brocklehurst, 2003). Many fat spreads may allow growth of yeasts, molds, spoilage bacteria (e.g. Enterobacteriaceae, pseudomonads, aerobic spore-formers) and even pathogenic bacteria when present or introduced during production or open shelf-life. Similar molds strains are spoilage agents of butter, fat spreads and margarine. Table 1.2. shows the microbiological guidelines developed for margarine (Uyttendaele et al., 2018).

Table 1.2. Microbiological guidelines for margarine (Uyttendaele et al., 2018)

Parameter	Target (CFU/g)	Tolerance (CFU/g)	Use by date/Best before date (CFU/g)
Yeasts	$< 1 \times 10^2$	5×10^2	3×10^5
Xerophilic molds	$< 1 \times 10^2$	5×10^2	No visible mold formation
Enterobacteriaceae	$< 1 \times 10^2$	5×10^2	5×10^2
Escherichia coli	$< 1 \times 10^1$	$< 5 \times 10^1$	$< 5 \times 10^1$
Coagulase-positive staphylococci	$< 1 \times 10^2$	$< 5 \times 10^2$	$< 5 \times 10^2$
Salmonella spp.	Absence in 25 g		
Listeria monocytogenes	Absence in 25 g	Absence in x g or $<1 \times 10^2$/g	1×10^2

However, the increased attention to hygiene during manufacturing, the quality of raw materials and the applied pasteurization conditions have all contributed significantly to product and process designs with a very good safety and spoilage record (Mostert and Lelieved, 2000). Nevertheless, several recalls have been reported of butter due to the presence of *Listeria monocytogenes*, often linked with raw milk butter and/or artisanal producers (Verraes et al., 2015).

1.3.1. Bacteria in food W | O emulsions

The recognition that pathogens are capable of surviving and growing in W | O emulsions such as butter raises interest in knowing their behavior in these products (Holliday and Beuchat, 2003). Food poisoning caused by consumption of blended margarine and butter products contaminated by *Staphylococcus intermedius* occurred in the United States in 1991 and involved over 265 people (Khambaty et al., 1994). A recall of a batch of Bandon Co-Op butter was performed in Ireland in 2019 after determining the presence of *Listeria monocytogenes* (FSAI, 2019). *L. monocytogenes,* VTEC and *S. aureus* have been identified as microbiological hazards in raw milk butter (Verraes et al., 2015). Sims et al. (1969) reported that butter made from inoculated cream supported growth of *Salmonella* at 25 °C, while populations decreased during storage below 4.4 °C. Cirigliano and Keller (2001) surface inoculated different commercial margarine and reduced-fat spreads with *L.monocytogenes* and found no growth for 7 days during storage at 5 and 23 °C. There are also reports showing growth of Listeria in butter. Voysey et al. (2009a) investigated the effects of butter characteristics on the growth of *Listeria monocytogenes* and determined that it grew better in coarse compared to fine butter. Interestingly, the growth at 8 °C was comparable to that at 21 °C.

Holliday et al. (2003) investigated the survival and growth of mixtures of five strains of *E. coli* O157:H7, five serotypes of *Salmonella* spp. and six strains of *L. monocytogenes* in 3 types of commercial butter: sweet cream whipped salted butter (pH 6.4; 78% fat), sweet cream whipped unsalted butter (pH 4.51; 78% fat) and salted light butter (pH 4.58; 43% fat, with preservatives). The products were subjected to temperature abuse (37 °C) and high relative humidity (85%) for 1 h to induce condensation of water on the surface, before storing at 4.4 °C or 21 °C for up to 21 days. Sweet cream whipped salted butter supported surface growth of all three pathogens at 21 °C and of only *L. monocytogenes* during storage at 4.4 °C. The other two products did not support growth of any of the three pathogens at either temperature for 21 days. All pathogens tested were inactivated more rapidly in products stored at 21 °C than at 4.4 °C and in products containing preservatives and acidulants. In a related study, Holliday and Beuchat (2003) showed that inactivation rates of pathogens in commercial yellow-fat spreads products vary according to product formulation (i.e. pH, emulsion characteristics, salt, fat and preservatives content) and storage temperature.

Lipolytic bacteria belonging to Micrococcaceae, Pseudomonaceae and *Bacillus* spp. may grow in coarse or unstabile W|O emulsions. Psychrotrophic Gram-negative bacteria such as *Pseudomonas* spp. and *Flavobacterium* spp. may develop and cause off-odor formation and rancidity (Driessen, 1983; Jooste et al., 1986; Champagne et al., 1993). Sometimes, spoilage may be caused by Enterobacteriaceae such as *Enterobacter* spp., usually as a result of a post-pasteurization contamination (ICMSF, 2005). Growth of *Shewanella putrefaciens* or *Flavobacterium malodoris* may lead to surface taints (Foster et al., 1957; Jooste et al, 1986) accompanied by the development of a putrid, decomposed or cheesy flavor. Certain strains of *Pseudomonas* spp. are associated with the formation of fruity odors or black discolorations of butter (Foster et al., 1957). In case of suspected spoilage problems, fat spreads should be tested for lipolytic microorganisms on media like the Eijkman-plate or Tributyrin agar (Tuynenburg Muys and Willemse, 1965).

1.3.2. Yeast and molds in food W|O emulsions

Yeasts and molds are important spoilage microorganisms of W|O emulsions and can result in surface discoloration and off-flavor (Varnam and Sutherland, 1994; van Zijl and Klapwijk, 2000). Some yeasts are lipolytic (*Candida lipolytica)* and can grow in W|O emulsions in the presence of high concentrations of salt, at low pH and at low temperatures (ICMSF, 2005). *Candida* (teleomorph *Pichia) guilliermondii* was isolated from butter samples on Pakistan market (Mushtaq et al., 2007). In retail samples of butter, *Rhodotorula* spp. *Saccharomycopsis*

lipolytica, Cryptococcus laurentii and *Candida diffluens* were reported as the predominant yeasts (Fleet and Mian, 1987).

Mold growth is the major stability problem associated with W│O emulsions. The water phase composition (i.e. salt level, pH, preservatives) affects mold growth and fine emulsions seem less likely to spoil (Brocklehurst, 2003). Conditions favoring mold development include high storage temperatures (>10 °C), bad packaging hygiene and development of free moisture on the outer surfaces of the product. The oil phase is virtually sterile due to the high temperatures applied during refining. Microbiological problems can be prevented by keeping the oil storage and transport systems free from water. As water is heavier than oil, water condensate will accumulate at the bottom of tanks or in low insufficiently flushed parts of process equipment which might create undesirable problems with molds (Brocklehurst, 2003).

Many mold species (*Alternaria* spp., *Aspergillus* spp*., Cladosporium* spp., *Geotrichum candidum, Paecylomyces variotti, Penicillum expansum, Penicillum roqueforti, Trichoderma viride, Trichoderma harzianum)* have been associated with W│O emulsion spoilage (Beerens, 1980; Hocking, 1994). Several sources state that molds grow through the fat matrix of emulsions, although no experimental data was stated (Brocklehurst, 2003; ICMSF, 2005; Erkmen and Bozoglu, 2016). Macy (1929) conducted an extensive review of the available literature on mold growth in fat matrices and found that pure fat is not available for direct use by molds and that sufficient quantities of water and nutrients appear to be necessary before the metabolization and utilization of the fat can be accomplished.

1.4. Food structure and microbial growth

The safety and quality of foods depend to a large degree on the extent to which they support microbial growth. Many food products are highly structured multiphasic systems, in which the conditions relevant to microbial growth can vary with position of the microorganisms in the food microstructure (Robins and Wilson, 1994). The effects of food structure on microbial growth include constraints on the mechanical distribution of water (Hills et al., 1996; 1997), the chemical redistribution of organic acids including food preservatives (Brocklehurst and Wilson, 2000) and physical constraints on mobility of microorganisms (Mattila and Frost, 1988; Dodd, 1990; Robins et al., 1994; Robins and Wilson, 1994; Wimpenny et al., 1995). Microorganisms occupy the water phase of foods. Microbial growth in a liquid water phase in food is usually planktonic and motile strains can move towards preferred regions of the food. Transport of nutrients to the bacteria and the release of metabolites result in a locally uniform environment until bulk chemical changes occur, such as a decline in pH (Wilson et al., 2002).

Often, the water phase of food is structured. This could be due to the addition of gums and thickeners to increase the bulk viscosity of foods (e.g. sausages) or the denaturation of protein to form protein micelles (e.g. cheese). In gelled regions of foods, microorganisms are immobilized and grow in the form of colonies (Dodd, 1990, Katsaras and Leistner, 1991, Wimpenny et al., 1995, Parker et al., 1998).

Studies in model experimental O|W emulsions have postulated a relationship between the concentration of oil in the emulsion and the form of microbial growth (Parker et al., 1995). When the oil concentration remained below 80% v/v the oil droplets were freely dispersed throughout the water phase and microbial growth was typically planktonic. At greater concentrations of oil phase, the oil droplets were close-packed, mobility of bacteria was prevented and growth was constrained as colonies which formed between oil droplets and displaced the droplets as the colonies expanded. Restriction of growth in this manner could result in a decline in growth rate and yield (Brocklehurst et al., 1995, Parker et al., 1995).

In W|O food emulsions,. growth of microorganisms is confined to within the water droplets which limit the availability of water, space and nutrients for growth (Verrips and Zaalberg, 1980; Charteris, 1995). The models of Verrips and Zaalberg (1980) and Verrips et al. (1980) are used to mechanistically predict the potential for growth of bacteria within discrete droplets of W|O emulsions related to the dimensions of the occupied droplets and the energy demands of the bacteria. The models show that bacterial growth and survival is restricted when the W|O emulsion microstructure remains intact and when coalescence of the droplets does not occur. This was confirmed in model experimental systems where an increase in numbers of bacteria in W|O emulsions was always accompanied by coalescence of the droplets of water phase (Brocklehurst et al., 1993).

The simplest form of microstructure affecting the growth of microorganisms is surface growth which typically results in the formation of colonies. Diffusion limitations are greater on a surface than within a matrix and depletion of oxygen and accumulation of protons immediately beneath the colony and extending into the substratum can result in decreased growth rates (Wimpenny and Coombs, 1983; Peters et al., 1987; Robinson et al., 1991). Comparisons of growth rates of surface colonies of *Salmonella* Typhimurium with the growth rate of cells immobilized in gel and with those growing planktonically in liquid medium followed the order: broth>immersed colonies>surface colonies (Brocklehurst et al., 1997).

1.4.1. Structure of food W | O emulsions

Food W | O emulsions are composed of liquid oil, fat crystals and an aqueous phase. The internal aqueous phase is dispersed as discrete spherical or irregularly shaped droplets within an outer oil phase, which may contain a mixture of fluid and crystalline fats. The fat crystals in these products give the product the required consistency and stabilize the water droplets. Because of the wettability of fat crystals, part of the solids are present in the W | O interface and influence the stability of the emulsion. (Juriaanse and Heertje, 1988). Figure 1.2. is a schematic drawing of the microstructure of butter (left) and margarine (right) at ambient temperature (Fuquay et al., 2011). The images are not entirely representative, as they are missing a representation of a more intricate solid fat crystal network (UGent internal communication).

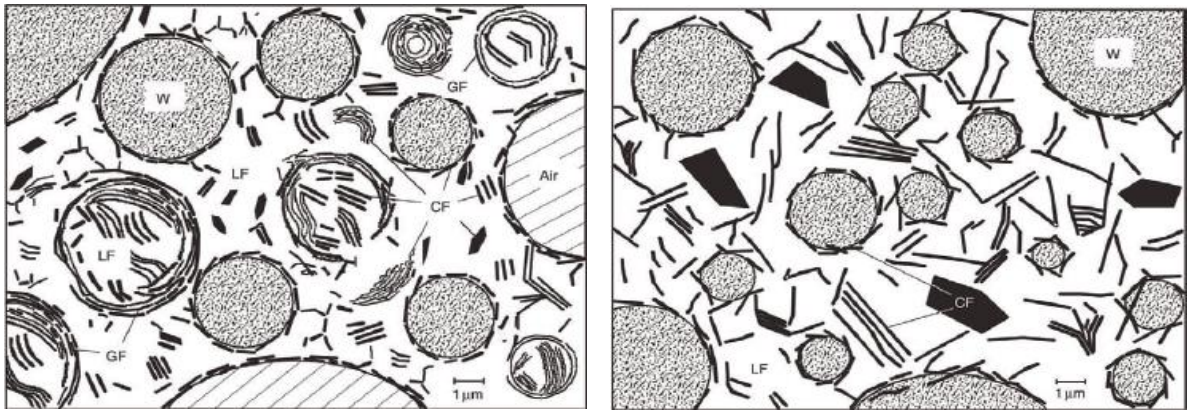


Figure 1.2. Schematic view of two W | O emulsion microstructures at ambient temperature: Butter (left); margarine (right). F – fat, W – water phase, CF – crystallized fat, GF – globular fat, LF – liquid fat (Fuquay et al., 2011)

The crystallization of melted lipids is the process of phase transition of molecules from liquid to solid state; the driving force is the difference between melting point of the fat and actual solution temperature (Grall and Hartel, 1992). The formation of texture in W | O emulsions is the result of crystallization of triacylglycerols with high melting points. The crystals do not behave as single components, but show different aggregation patterns with formation of a three-dimensional fat crystal network. Fat spreads derive their consistency from the fat crystal network (Juriaanse and Heertje, 1988). In lipids, three specific types of fat crystals are predominant, the polymorphs α, β′ and β (Ribeiro et al., 2015). Form α is metastabile, with hexagonal chain packing. Form β′ has intermediate stability and orthorhombic perpendicular packing, while form β has greater stability and triclinic parallel packing (Martini et al., 2006). The transition from the α form to the β′ form takes place in the crystallization equipment. Most fats tend to crystallize in the β′ form when crystallization takes place in an SSHE (scraped

surface heat exchanger) plant (Kirkeby, 2006). This transformation will only take place in the direction of a more stabile form seeking the most compact crystal form and the lowest thermodynamic state possible (Sato, 1988). Of all modifications the β modification has the highest ordering and consequently the highest melting point. However, the β′ modification is dominant in food W│O emulsions due to blending different raw materials with the aim of arriving at the desired overall crystallization and melting behavior (Juriaanse and Heertje, 1988).

Microscopic and nuclear magnetic resonance (NMR) spectroscopy methods have been developed to characterize the water dispersion in fatty emulsions. Such examinations demonstrated that, in properly produced butter, the water phase is almost exclusively present as isolated, globular or elongated droplets with diameters < 30 µm, covered with high-melting butter fat crystals (Buchheim and Dejmek, 1990). The more work is put into making the butter, the smaller the droplets are and the finer their distribution is (Voysey et al., 2009). Discrete water droplets are covered by thin fat crystal plates densely arranged at the W|O interface (Fuquay et al., 2011). Proteins can be added to the water phase where they will contribute to water phase viscosity and water holding capacity (Charteris, 1995).

Verrips and Zaalberg (1980) claimed that in the case of margarines the droplets of water phase are typically irregular in shape and can range between 0.3 and 30 µm in diameter. The shells of fat crystals that cover the water droplets prevent these from merging together (Juriaanse and Heertje, 1988).

The structure of reduced-fat spreads is generally similar to that of butter or margarine, although the water phase may be structured by added thickeners and larger water droplets may occur that make the water dispersion coarser (Keogh et al., 1988; Madsen, 1990; van Zijl and Klapwijk, 2000). Emulsion characteristics for a 40% fat spread containing protein are, for example, 50% of the water volume in droplets with diameters < 15 µm and less than 5% in droplets with diameters > 90 µm. Reduced-fat spreads lacking protein are easier to produce and often have a finer water dispersion, e.g. 50% of droplets have a diameter <10 µm and < 5% of droplets have a diameter > 45 µm. Fat-continuous spreads containing 20% fat can have a very coarse water dispersion, e.g. 50% of droplets have a diameter < 50 µm and < 5% of droplets have a diameter > 300 µm (ICMSF, 2005).

1.4.2. Influence of W|O emulsion structure on microbial growth

Foods are often non-homogenous matrices. The structure of the food creates local chemical or physical environments that affect the spatial distribution, survival and growth of microorganisms (Wilson et al., 2000).

Strategies to prevent or retard microbial growth in emulsions include adjustment of the chemical formulation of the products and control of the storage temperature. These measures apply both to O|W food emulsions (e.g. salad creams and mayonnaise) and to the inverse W|O emulsions (e.g. margarines and low fat spreads) (Brocklehurst et al., 1993). Initially, it was assumed that, within the food matrix, it is the chemical composition alone that controls microbial growth. The experimental basis for this paradigm was the growth of microorganisms in liquid culture medium. The assumption was that microorganisms would grow similarly in liquid culture medium as they would in foods of an equivalent chemical composition, but this approach disregarded the effect of structure of food on microbial growth (Brocklehurst et al., 1993: Robins and Wilson, 1994; Robins et al., 1994).

Studies of microbial growth in food emulsions usually involve deliberate inoculation of industrially produced products, where growth is characterized as a function of temperature and chemical composition of the emulsion (Notermans et al., 1993). Often, microbiological studies lack physical characterization of emulsions so information concerning the effect of emulsion structure on the growth of bacteria is rarely available. In addition, the methods used to recover microorganisms from emulsions include dilution, mixing and other pre-treatments (e.g. Gomez-Lucia et al., 1990; Hale et al., 1990) and information regarding the method of inoculation, morphology and site of growth of bacteria is missing.

In W|O emulsions, the physical entrapment and dispersion of the water phase as discrete droplets in a lipid matrix restricts microbial growth to a small number of droplets which can provide limited nutrients and in addition possess antimicrobial substances. The W|O character of fat spreads gives an important contribution to their microbiological stability especially after they have been exposed to microorganisms commonly found in a domestic environment. Their microbiological stability is influenced by the concentration and physical nature of the continuous lipid phase with product stability increasing with greater proportions of crystallized fat (Charteris, 1995).

Some parameters that describe emulsion characteristics are the volume weighted mean diameter - D_{43} (μm) and the geometric standard deviation (e^{σ}) of the droplet size distribution

(DSD) (Alderliesten, 1990; 1991). A fat spread with a lower D_{43} but a higher e^{σ} can be made up of a larger number of large volume droplets, which adds to the mold sensitivity of the product. The contribution of e^{σ} to mold stability will be reduced, if D_{43} increases. At higher D_{43}, the volume fraction of droplets prone to mold spoilage will hardly increase when e^{σ} goes up (ter Steeg et al., 2001).

When full-fat food W|O emulsions such as margarine and butter have a moisture content of 16% by weight, they contain approximately 10^7 water droplets per mL. Low-fat food W|O emulsions with 50% moisture by weight contain approximately 10^{12} droplets per mL. When the size and number of water droplets are compared with the average size and total count of microorganisms that may be encountered immediately post-manufacture, it appears that the majority of moisture droplets in food W|O emulsions are sterile. The dimensions of bacteria typically range from between approximately 0.4-1.5 µm by 0.5-5 µm for bacilli and approximately 0.5-2.0 µm in diameter for cocci (Charteris, 1995). Boysen (1927) concluded that moisture droplets of less than 10 µm in diameter are too small to permit microbial growth.

Keogh et al. (1988) stated that there are several differences between an 80% fat and a 40% fat W|O emulsion: (i) the water content of the 40% fat product is higher and salt and other water-soluble preservatives (e.g. organic acids) are diluted to such an extent that their microbiologically inhibitory effect could be reduced. This cannot be compensated for by adding more salt or acid because of sensory deviations. (ii) In order to structure the water phase of the 40% fat product, biopolymers are added (thickeners and vegetable or animal protein), which may increase the microbiological vulnerability of the water phase. (iii) The water droplet size distribution of the 40% fat W|O emulsion usually has a higher D_{43} and a larger distribution width (e^{σ}) compared to an 80% fat W|O emulsion. As a consequence, reduced-fat food W|O emulsions are more vulnerable to microbiological problems than full-fat food W|O emulsions.

If the aqueous phase in food W|O emulsions is compartmentalized in sufficiently small droplets that are well dispersed throughout the product, proliferation of microorganisms is limited by lack of space or by exhaustion of the nutrients in the droplets. Microbial growth occurs similarly in butter and margarine, although the former may have a coarser structure (Jensen et al. 1983, ICMSF 2005). The presence of proteins in the water phase of W|O emulsions provides a source of amino acids for microbial proliferation (Precht and Buchheim, 1980; Keogh et al., 1988). The rate and extent of growth depends on the microflora present and the storage temperature, with temperatures in excess of 10 °C facilitating relatively rapid microbial growth and product deterioration (Charteris, 1995).

A model to describe quantitatively the growth of microorganisms in W│O emulsions based on a function of the initial contamination and the numbers of droplets exceeding the minimum size for occupancy, has been developed (Verrips and Zaalberg, 1980). Verrips et al. (1980) demonstrated that bacteria in W│O emulsions were confined to very few of the isolated and non-coalescing droplets. Their growth was predicted to cease when the limited quantity of nutrients within the droplet was exhausted. This was expanded further by modelling the energy demands of the contained bacteria (ter Steeg et al., 1995). The model quantitatively describing microbial growth in W│O emulsions proposed by Verrips and Zaalberg (1980) was based on several factors. First, the size distribution of water droplets measured by NMR analysis. The second factor taken into account was the distribution of microorganisms over water droplets in the W│O emulsion. It was assumed that the microorganisms are distributed over all droplets with a radius equal to or larger than half the longest axis of the microorganism considered, r(m). It was also assumed that the distribution of microorganisms over droplets with a certain radius, r, is proportional to the total volume of droplets with this radius, $V_{tot}(r)$, divided by the total volume of all droplets with a radius r larger than r(m), $V_{tot}(r>r(m))$. This was experimentally proven by two approaches. First, emulsions were made with radioactive glucose (C_{14}) and inoculated with high numbers of *Citrobacter freundii* (10^7 microorganisms per gram). The emulsions were analyzed at regular intervals for the number of cells, radioactive acids formed and the amount of radioactive glucose remaining. The results of the survival of the bacterium in emulsions were used to determine the maximum occupation of a droplet with microorganisms. For all experiments, this value was about $4x10^9$/mL. Using that information, the minimum radius allowing for a water droplet to be occupied by a microorganisms was varied until the measured and calculated data fitted well. A drawback of this method was that, in products with an initial contamination of $>10^7$ microorganisms, (nearly) all large droplets contained a microorganism and consequently the presence of microorganisms in smaller droplets increased. Therefore the r(m) obtained in this way was most probably smaller than the real radius of the smallest droplet occupied with microorganisms for products with a low contamination. Second, W│O emulsions with relatively high numbers of microorganisms ($N > 10^6$) had been prepared and the distributions of microorganisms over the droplets had been determined microscopically. The results of these experiments showed clearly that the microorganisms were distributed over the droplets proportionally to the volume of all droplets with that radius, i.e. the more droplets there were with radius r, the more droplets with that radius were occupied with microorganisms. The third factor taken into account was the Monod relation between the amount of nutrients available in the water droplet and the biomass formed. They demonstrated that, in poor water phases, with a low concentration of growth limiting nutrients (e.g. 1×10^{-9} µg/µm^3), it is the nutrient concentration that limits the number of microbial cells after maximum growth. However, in rich water phases, with a high concentration

of nutrients (e.g. 1×10^{-8} µg/µm^3), it is the spatial limitation of the water droplet that governs the maximum number of bacterial cells formed, no matter the size of the minimum droplet supporting growth. The amount of nutrients present in one water droplet is proportional to the size and volume of that droplet. The fourth factor taken into account was the exclusion of the possibility that water-soluble nutrients diffuse through the lipid barrier. It was mentioned that the average lipid layer around the water droplet in an 80% fat emulsion is about 3.0 µm and this thickness would exclude the diffusion of nutrients like sugars or amino acids.

It was also observed that the growth of selected lipolytic micrococci and yeasts was strongly limited in a compartmentalized system. Although a clear explanation wasn't given, it was suggested that two factors were involved: the amount of lipase formed by the few cells in a droplet were too small to provide enough nutrients for further growth and/or the hydrolysis of lipids by lipases is inhibited by the emulsifier coating and the physical structure of the fat. The synergism between several chemicals that have an inhibitory action and compartmentalization was evaluated (Verrips, 1989). For the microorganisms tested (Enterobacteriaceae, Micrococcaceae and various types of yeast) there was a strong synergism, as was also the case between compartmentalization and low temperatures. Whereas growth of lipolytic microorganisms in W│O emulsions seemed to be well inhibited by the compartmentalized nature of the product, it was claimed that the growth of molds on the surface of W│O emulsions was only partly inhibited, stressing again that both nutrient and space limitation are the stabilizing factors in compartmentalized products.

Brocklehurst et al. (1993) investigated the growth of bacteria in model W│O emulsions, where the lipid phase was liquid. They claimed that, at the time, there was a move among manufacturers of food W│O emulsions towards the use of a fluid, rather than solid fat phase. It was shown that the bacteria occupied a small proportion of the water phase droplets (Figure 1.3a) and their growth was not restricted by the size of the droplets that they occupied. Growth of bacteria was accompanied by coalescence of droplets which supplied further space, nutrients and water for the bacteria to grow to very large numbers and eventually occupy enormous droplets (Figure 1.3b). In addition, the solubility of oxygen in many oils used in the manufacture of emulsions is several times greater than the solubility of oxygen in water. For example, the solubility of oxygen in sunflower oil is 5 times greater than in water (Cuvelier et al., 2017). This phenomenon is manifested in oxidative rancidity problems associated with edible vegetable oils, but the oxygen contained within them could equally provide a source of oxygen to the bacteria (Brocklehurst et al., 1993). From a microbiological point of view, a limitation of the closed shelf-life period of full-fat food W│O emulsions is usually not required. But, the shelf-life is usually limited to 3-6 months for reasons of chemical stability. In practice,

both preserved and non-preserved full-fat food W│O emulsions appear to be very stabile products during closed and open shelf-life. (ICMSF, 2005).

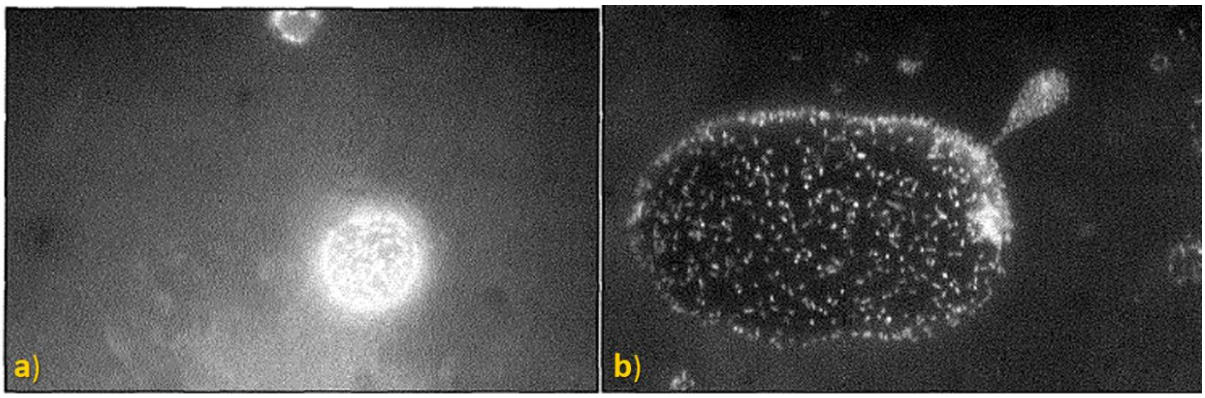


Figure 1.3. a) Bacteria occupying droplets of water phase in liquid W│O emulsions. b) Exhaustive growth of bacteria caused droplet coalescence (Brocklehurst et al., 1993).

As a continuation of the work of Verrips and Zaalberg (1989), ter Steeg et al. (2001) initiated the development of modelling mold behavior in W│O emulsions and assessed and modelled the influence of (natural) antimicrobials for shelf-life extension of W│O emulsions. 60% fat model W│O emulsions were made with reproducible droplet size distributions and the action of green antifungals and lytic enzymes against *Penicillium roqueforti* (a key spoilage mold) and *Trichoderma harzianum* was tested.

Lipid phase of the 60% fat W│O emulsions was comprised of sunflower oil to which a 10% solution of triacylglycerol fat crystals in sunflower oil was added. An emulsifier comprising of 90% monoglycerides in sunflower oil was added to the lipid phase (2750 mg/kg on total emulsion). Water phase was comprised of 15 g/L potato dextrose extract, 10 g/L malt extract, 50 mM citric acid and buffered at pH 4.85. A range of varying droplet sizes was obtained by varying stirring speed during W│O emulsion preparation. Only a narrow range of droplet sizes could be achieved by varying stirring speeds, higher ranges were achieved by mixing emulsions of different D_{43}. Green antifungals chosen were carvacrol, dihydrocarveol and undecanol. Antifungal activity of lytic enzymes was assessed using Novozym 234 which is an enzyme cocktail produced by *T. harzianum.* Activity of Novozym 234 was tested on *Paecilomyces variotti*, a mold particularly sensitive to cell wall degrading enzymes (Brul et al., 1997). The effectiveness of green antifungals was compared with the inhibitory action of sorbic acid. *P. roqueforti* was selected because of its capability to adapt and grow in the presence of high levels of undissociated sorbic acid (> 300 mg/kg $(HA)_{aq,eq}$) (Liewen and Marth, 1985). The W│O emulsions were inoculated with 10-100 spores/g of the fore mentioned molds, incubated at 10 and 20 °C and sampled according to the expected vulnerability of the water phase up to 9 months.

Novozym 234 was ineffective to prevent outgrowth of *P. roqueforti.* All antimicrobials had significant effect on time to visual spoilage and $(HA)_{aq,eq}$ was clearly inhibitory. 170 mg/kg $(HA)_{aq,eq}$ was only able to prevent growth in emulsions with a $D_{43} < 9.5$ µm. The results indicated that a low concentration of $(HA)_{aq,eq}$ has a large impact on the time to visible spoilage, especially at elevated temperatures. Temperature had a large antifungal effect only if there was no sorbic acid present in the system. Other than D_{43} which is generally used as a quantitative parameter for microbial susceptibility of emulsions, the emulsion droplet size distribution was captured in a novel mechanistic parameter DSD-I (Droplet Size Distribution Influence). DSD-I is a combination of available water droplets whose volume is bigger than the minimal (threshold) diameter for supporting fungal germination and outgrowth, the mean volume of the water droplets and specific surface area of the droplets to initiate and sustain fungal outgrowth. Experimental validation of predictive models showed that using D_{43} and distribution width e^{σ} instead of DSD-I gave better results for emulsions with high e^{σ} (more vulnerable emulsions). While assessing the interaction of green antifungals and DSD-I it was concluded that the antimicrobials gave a minor but significant shelf life extension, however, their effect was lost at a higher DSD-I.

1.5. Behavior of sorbic acid in food W | O emulsions

1.5.1. Sorbic acid and its salts

Sorbic acid and its salts have become one of the leading preservatives in the food sector throughout the world because of their physiological inertness, effectiveness even in the weakly acid pH range and neutral taste (Lück, 1990). Sorbic acid is a straight-chain, trans-trans unsaturated fatty acid (2,4-hexadienoic acid; CH3-CH=CH-CH=CH-COOH), with a molecular weight of 112.13 g/mol. The carboxyl group of sorbic acid is highly reactive and results in formation of various salts and esters (Sofos, 1989). Sorbic acid (E200) appears as white crystals and is used in its acid form or as sodium (E201), potassium (E202) or calcium (E203) salts, which are white powders (Eklund, 1989). Sorbic acid is sparingly soluble in water (0.16 g/L), calcium sorbate and sodium sorbate exhibit better water solubility (10.2 and 320 g/L, respectively) and potassium sorbate shows good solubility (582 g/L) and can be used to produce 50% stock solutions (Lück, 1990).

Effective antimicrobial concentrations of sorbic acid in most foods are in the range of 0.02 to 0.30% (Stopforth et al., 2005).

Sorbic acid and its salts inhibit various bacteria, including sporeformers, at various stages of their life cycle (germination, outgrowth and cell division) (El-Shenawy and Marth, 1988; Zhao et al., 1993; Kouassi and Shelef, 1995a,b; Sofos, 2000; Koodie and Dhople, 2001). Bacterial species inhibited by sorbic acid belong to the genera *Acetobacter, Achromobacter, Acinetobacter, Enterobacter, Aeromonas, Alcaligenes, Alteromonas, Arthrobacter, Bacillus, Campylobacter, Clostridium, Escherichia, Klebsiella, Micrococcus, Moraxella, Mycobacterium, Proteus, Pseudomonas, Salmonella, Serratia, Staphylococcus, Vibrio, Yersinia* and others. This multiple action of sorbate may be responsible for its broad effectiveness compared to other antimicrobial agents (Sofos, 1989).

Extensive research during the 1950s demonstrated the effectiveness of sorbic acid against yeasts and molds and resulted in the extensive use of the compounds as fungistatic agents in many foods. The effectiveness of sorbic acid against yeasts has been documented by numerous studies (Emard and Vaughn, 1952; Ferguson and Powrie, 1957; Geminder, 1959; Pederson et al., 1961; Huang and Armstrong, 1970; El Halouat et al., 1998; Bracey et al., 1998; Piper et al., 1998, Šoljić et al., 2018). Yeasts inhibited by sorbic acid include species of the genera *Brettanomyces, Candida, Cryptococccus, Debaryomyces, Endomycopsis, Hansenula, Kloeckera, Pichia, Rhodotorula, Saccharomyces, Sporobolomyces, Torulaspora, Torulopsis* and *Zygosaccharomyces* (Sofos, 1989).

Numerous studies have also documented the effectiveness of sorbic acid against molds (Emard and Vaughn, 1952; Deuel et al., 1954a,b; Melnick and Luckmann, 1954a,b; Melnick et al., 1954a,b; Smith and Rollin, 1954a,b; Huang and Armstrong, 1970; Baldock et al., 1979; Kaul et al., 1979; Kivanç, 1992; Garza et al., 1993; Skirdal and Eklund, 1993; Aly, 1996; Fan and Chen, 1999). Mold species inhibited by sorbates belong to the genera *Alternaria, Ascochyta, Ascosphaera, Aspergillus, Botrytis, Cephalosporium, Chaetomium, Cladosporium, Colletotrichum, Cunninghamella, Curvularia, Fusarium, Geotrichum, Gliocladium, Helminthosporium, Heterosporium, Humicola, Monilia, Mucor, Penicillium, Phoma, Pepularia, Pestalotiopsis, Pullularia, Rhizoctonia, Rhizopus, Rosellinia, Sporotrichum, Trichoderma, Truncatella, Ulocladium* and others. Sorbic acid inhibits molds in butter, sausages, fruits and juices, cakes, grains, bread and smoked fish (Chichester and Tanner, 1972; Liewen and Marth, 1984, 1985; Sofos, 1989).

1.5.2. Mechanism of antimicrobial action of sorbic acid

Sorbic acid concentrations used in food preservation (< 0.3% on total product mass) inhibit or delay growth of vegetative microorganisms without causing permanent or lethal damage to

their cells, while higher concentrations may cause cell inactivation. Several mechanisms of the inhibition of metabolic activity of certain microorganisms by sorbic acid have been proposed (Stopforth et al., 2005; EFSA, 2015).

Inhibition of microbial cell metabolic function by sorbic acid may result from alterations in cell membranes and cell transport functions, inhibition of enzymes involved in transport function or metabolic activity, alteration of the morphological structure of the cell, or changes in the genetic material. This multiple action of sorbate may be responsible for its broad effectiveness compared to other antimicrobial agents (Sofos, 1989). One mechanism proposed for the inhibitory action of sorbic acid is the excessive consumption of cellular energy that occurs as a consequence of the cell eliciting a stress response while attempting to maintain pH homeostasis (Bracey et al., 1998). Inhibition of microbial proliferation by sorbic acid may be due to neutralization of the transport driving proton-motive force that exists across cell membranes. Protons and hydroxyl ions are separated by the cytoplasmic membrane, with protons that are outside the cell giving rise to acidic pH and the latter, inside the cell, giving rise to pH near neutral. The membrane gradient created represents electrochemical potential that the cell employs in the active transport of some compounds such as amino acids. Weak lipophilic acids act as protonophores. This means that after diffusing across the membrane, the undissociated molecule ionizes inside the cell and lowers intracellular pH. This, in turn, results in a weakening of the transmembrane gradient so amino acid transport is adversely affected (Jay et al., 2005). Sorbic acid is known to inhibit the *in vitro* activity of many enzymes, especially sulphydryl-containing enzymes. Although exact mechanisms of inhibition and inactivation of specific enzymes are unknown, indirect evidence supports the theory that binding of sorbic acid with sulfhydryl groups inhibits their activity (Kouassi and Shelef, 1995a, b). Inhibition of the enzyme catalase was attributed to autoxidation of sorbic acid and formation of sorbyl peroxide. It has also been suggested that sorbic acid combines with coenzyme A forming sorbyl coenzyme A, which causes microbial inhibition through interference with oxygen uptake (Sofos et al., 2005). Information on the effect of sorbic acid on the genetic material of microorganisms is limited. However, the compound is believed not to possess mutagenic activity, but it may form mutagenic products when reacting under certain conditions with compounds such as sulfur dioxide and sodium nitrite (Naidu, 2000).

Sorbic acid was found to inhibit many bacterial strains such as *Enterobacter*, *Bacillus*, *Campylobacter*, etc. Inhibition of bacteria appears to cause an extension of the lag phase, with a lesser influence on rate and extent of growth. Under certain conditions, some microbial strains are resistant to sorbic acid or even metabolize the compound (Sofos, 1989). As for bacterial spores, sorbic acid has been described as a competitive and reversible inhibitor of

amino acid inducted bacterial spore germination by acting on the connecting reactions following the initiation of germination. Indirect evidence suggests that inhibition could involve alteration of permeability of spore membranes or inhibition of spore lytic enzymes that may be involved in germination by causing cortex hydrolysis and loss of refractility (Blocher & Busta, 1985; Sofos et al. 2005).

Sorbic acid has been reported to cause morphological changes in yeasts with development of yeasts cells with dense lipoprotein granules, irregular nuclei, numerous mitochondria of various sizes and vacuoles (Naidu, 2000).

Several studies have documented the effectiveness of sorbic acid in molds (Chichester and Tanner, 1972). Sorbic acid inhibits conidial germination and mycelial growth of *A. niger* through intracellular acidification (Plumridge et al., 2004). The inhibition of *Penicillium roqueforti* by sorbic acid was found to be associated with a change in the composition of phospholipids and of neutral lipids, as well as the fatty acid composition of fungal lipids (Sergeeva et al., 2009). Sorbic acid was found to inhibit the formation of mycotoxins by various molds in culture media and in foods (Bullerman, 1983, 1984, 1985). However, sub-inhibitory levels of sorbic acid may stimulate the production of mycotoxins (Bullerman and Olivigni, 1974). Sorbic acid has been reported as an inhibitor of mold growth through the depletion of ATP levels in conidia. This could potentially take place because ATP levels may be depleted as the cell attempts to maintain ion balance when dissociation of sorbic acid in the cytoplasm increases the intercellular cation concentration and because the primary sodium/hydrogen pump is directly linked to hydrolysis of ATP. As the hydrogen influx exceeds the pumped efflux, a shift in charge may potentially take place and lead to a decrease in the net negative intercellular change. This could then discharge the pH gradient required for ATP formation according to the chemostatic theory of oxidative phosphorylation (Sofos et al., 2005). Marth et al. (1966) conducted systematic studies with molds in cheese and reported that certain mold species degraded sorbic acid and that sorbic acid degradation was enhanced by a nutritious substrate and retarded by a poor medium. The degradation of sorbate was accompanied by the formation of a volatile compound with a hydrocarbon-like odor, which was identified as 1,3-pentadiene. It was postulated that the molds degraded sorbic acid through decarboxylation.

Overall, sorbic acid is considered as a more effective inhibitor of yeasts and molds than of bacteria. The mechanism of inhibition or of delay of growth by sorbic acid is dependent on the microbial types, species, strains, substrate properties and environmental factors (Skirdal and Eklund, 1993, Sofos, 1989).

1.5.3. Sorbic acid in biphasic foods

The site of growth of microorganisms in emulsions is the aqueous phase and it is its chemical composition that has a direct influence upon the survival and growth of microorganisms (Brocklehhurst et al., 1993). However, the lipid component may have a controlling influence on microbial growth through its contribution of structure on the growth domain of the microorganisms and its contribution to the redistribution of chemical components between the phases of the foods (Brocklehurst and Wilson, 2000). The lipid phase of emulsions could decrease the concentration of weak acid preservatives within the water phase by partitioning and this may contribute significantly to high local numbers of bacteria in water droplets (Brocklehurst et al., 1993). In biphasic foods, which contain water and lipid phases, antimicrobial undissociated organic acids partition between the water and lipid components which decreases their concentration in the aqueous phase. This is because the antimicrobial undissociated form of organic acids is lipophilic (Lubieniecki-von Schelhorn, 1967; Leo et al., 1971). The pH of foods preserved using organic acids is typically in a region where weak organic acids are present in both the undissociated and the dissociated forms (Brocklehurst and Wilson, 2000).

The antimicrobial efficacy of organic acids is affected by the fundamental thermodynamic characteristics of dissociation and partition. However, these characteristics of the acids are also key for understanding the controlling effect of the lipid phase of emulsions on the growth of microorganisms (Brocklehurst and Wilson, 2000).

Dissociation is an important property of organic acids that influences their effectiveness as antimicrobial agents in emulsions. This is a characteristic of many food preservatives and it is the separation of a chemical compound into distinct component moieties, the undissociated and dissociated acid. In the case of food preservatives this is by an ionization reaction (Brocklehurst and Wilson, 2000). The dissociation equation for sorbic acid is shown in Equation 1.1.

$$C_6H_8O_2 \rightleftharpoons C_6H_7O_2^- + H^+ \qquad (1.1)$$

The dissociation process is an equilibrium and the concentration of each side of the equilibrium equation is dictated by the acid dissociation constant (or K_a). For convenience the negative logarithm of the acid dissociation constant, pK_a, is used. The values of K_a for acetic and sorbic acid at 25 °C are 1.76×10^{-5} ($pK_a = 4.76$), for lactic acid it's 1.37×10^{-4} ($pK_a = 3.86$) and for benzoic acid it's 6.46×10^{-5} ($pK_a = 4.20$). K_a is affected slightly by temperature (Reijenga et al., 2013). The K_a equation for sorbic acid is given in Equation 1.2.

$$K_a = \frac{[C_6H_7O_2^-][H^+]}{[C_6H_8O_2]} \quad (1.2)$$

When the pH of the emulsion is equal to the pKa of the acid, the concentration of undissociated acid is equal to the concentration of dissociated acid. As the pH of the emulsion decreases, the proportion of undissociated acid increases, conversely, as the pH of the emulsion increases then the proportion of undissociated acid decreases. This is important for the microbiological stability of these foods because it is the undissociated acid that is the predominantly antimicrobial form (Baird-Parker, 1980; Sofos and Busta, 1981; Eklund, 1983).

Table 1.3. The effect of pH on the concentration of undissociated sorbic acid, [HA].

pH	3.5	4.0	4.5	5.0	5.5	6.0	6.5
[HA] (%)	94.7	84.9	64.0	36.0	15.1	5.3	1.7

When emulsions are made, some of the sorbic acid dissolves in the lipid phase and is hence unavailable as an antimicrobial compound (Wilson et al., 2000). Figure 1.4. shows the dissociation of potassium sorbate in water and partitioning of sorbic acid into the oil phase

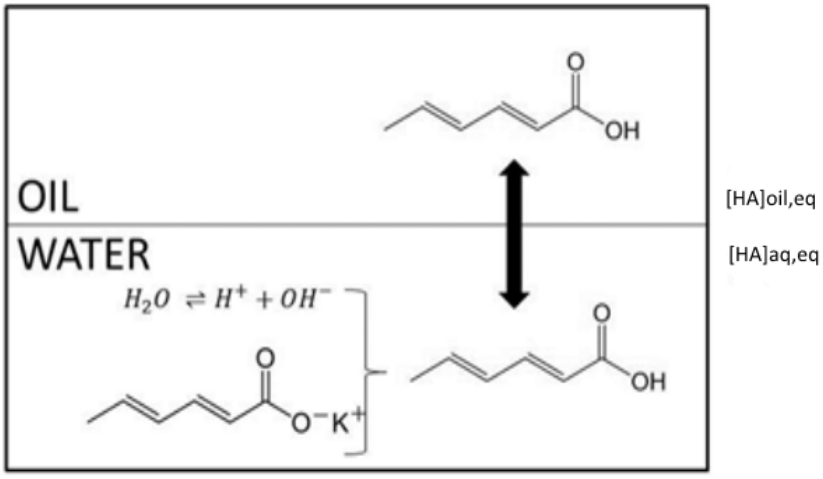


Figure 1.4. Potassium sorbate dissociation and sorbic acid partitioning behavior in a W|O emulsion.

1.5.4. Partition coefficient of sorbic acid

The extent of solubility in the lipid phase varies between organic acids and differs according to the type of oil used. This behavior is described by their partition coefficient, K_p. Partition

coefficients provide a way to test the accuracy of atomistic force fields in various solvent environments. They describe the ratio of concentrations of a neutral solute molecule in a system with two immiscible solvents;. This value differs from a distribution coefficient, $K_{d'}$ (also known as “apparent partition coefficient”) which includes all ionized and unionized forms of the solute (Bannan et al., 2016). Equation 1.3. shows the calculation for K_p between an oil and aqueous phase where $[HA]_{oil,eq}$ and $[HA]_{aq,eq}$ (mol/L) are concentrations of undissociated sorbic acid in the oil and aqueous phase at equilibrium, respectively. Table 1.4 shows the K_p values of sorbic acid reported in literature.

$$K_p = \frac{[HA]_{oil,eq}}{[HA]_{aq,eq}} \quad (1.3)$$

Table 1.4 Sorbic acid K_p values reported in literature.

Source	Matrix	Reported K_p
Lund et al. (2000) and ter Steeg et al. (2001)	Oil	3.3
Lück and Jager (1997)	Edible oil	3
Heintze (2002)	Octanol (pH 2.5, 20 °C)	21
	Octanol (pH 6.5, 20 °C)	0.02
Lubieniecki-von Schelhorn (1967)	Peanut oil (20 °C)	3.1
Lubieniecki-von Schelhorn (1967)	Sunflower oil (20 °C)	4.5
Lubieniecki-von Schelhorn (1967)	Soybean oil (20 °C)	3.0
Cheng et al. (2010)	Fish oil (25 °C)	3.03
Cheng et al. (2010)	Rape oil (25 °C)	4.19
Cheng et al. (2010)	Olive oil (25 °C)	3.65
Gibson (2016)	Oil	3.5

Sorbic acid has a favorable oil/water partition coefficient compared to, for example, benzoic acid (i.e. 6 to 13, reported by Wilson et al. (2000)), so in W|O emulsions a relatively high proportion of sorbic acid/sorbate remains in the water phase, which is the one primarily susceptible to microbial contamination and proliferation. Sorbic acid is added to the lipid phase and/or potassium sorbate to the water phase in appropriate quantities (Lück, 1980). In general, the partitioning of sorbic acid between the water and oil phases of foods depends on the pH of the food, the amount of oil and other ingredients present (Sofos, 1989). The water phase of many foods contains a range of solutes, commonly added for organoleptic purposes or, as in the case of NaCl and sucrose, to decrease the water activity of the food in order to increase its microbiological stability. However, the addition of solutes to the water phase can increase the partitioning of undissociated organic acids into the lipid phase of W|O emulsions. (Gooding et al., 1955; Sofos and Busta, 1981). This further decreases the concentration of the effective form of the antimicrobial in the water phase.

1.5.5. Extension of preservative distribution model by Wilson et al. (2000)

A modified form of the Henderson-Hasselbalch equation has been proposed (Wilson et al., 2000) which takes the partitioning effect of weak organic preservatives into account and gives the proportion of active (inhibitory) preservative in a biphasic system, given the pH, the volume fraction of oil and the partition coefficient of the undissociated weak acid (Equation 1.4).

$$[HA]_{aq,eq} = \frac{[HA]_{tot}}{1 + K_p \times \left(\frac{\varphi}{1-\varphi}\right) + 10^{(pH-pKa)}} \quad (1.4)$$

Where $[HA]_{aq,eq}$ (mol/L) is the active inhibitory form of weak organic acid present in the aqueous phase at equlibrium, $[HA]_{tot}$ (mol/L) is the total concentration of weak organic acid in the system, φ is the volume fraction of oil in the system (Equation 1.5) and K_p is the O|W partition coefficient of the weak organic acid (Equation 1.3).

$$\varphi = \frac{V_{oil}}{V_{oil} + V_{aq}} \quad (1.5)$$

Where V_{oil} and V_{aq} (L) are the volumes of the oil and aqueous phase, respectively.

Due to the low solubility of sorbic acid in water (0.16 g/L at 20 °C), it is industrially added in its acid form to the lipid phase of emulsions, in its salt form (usually potassium sorbate) in the aqueous phase or as a combination of the two aforementioned applications (Kirkeby, 2006). The mass balance defined in Wilson et al. (2000) describes adding $[HA]_{tot}$ in the aqueous phase of the biphasic product (Equation 1.6). Thus, it was first assessed if $[HA]_{aq,eq}$ expressed as a function of the initial concentration of sorbic acid in oil, $[HA]_{oil,i}$, would be comparable to Wilson et al. (2000) model.

$$[HA]_{tot}V_{aq} = [HA]_{aq,eq}V_{aq} + [A^-]_{aq,eq}\,V_{aq} + [HA]_{oil,eq}\,V_{oil} \quad (1.6)$$

Where $[A^-]_{aq,eq}$ and $[HA]_{oil,eq}$ (mol/L) are the concentrations of the base in the aqueous phase and undissociated sorbic acid in the oil phase at equilibrium, respectively. The term $[A^-]_{oil,eq}$ is excluded in the above mentioned equation because the weak acid does not dissociate in the oil phase.

A mole balance describing the addition of sorbic acid into the oil phase of a biphasic product can be written as follows:

$$\frac{m_{HA_{oil,i}}}{MW_{HA}} = n_{HA_{oil,i}} = [HA]_{oil,i}V_{oil} = [HA]_{oil,eq}V_{oil} + [HA]_{aq,eq}V_{aq} + [A^-]_{aq,eq}V_{aq} \quad (1.7)$$

$m_{HA_{oil,i}}$ (g) represents the mass of sorbic acid added to the oil phase, MW_{HA} (g/mol) is the molecular weight of sorbic acid (i.e. 112.13 g/mol) and $n_{HA_{oil,i}}$ (mol) represents the moles of sorbic acid present in the system.

Considering φ (Equation 1.5) and K_p (Equation 1.3), and dividing Equation 1.7 by V_{oil}, the following equation is obtained:

$$[HA]_{oil,i} = [HA]_{aq,eq}K_p + [HA]_{aq,eq} \times \left(\frac{1-\varphi}{\varphi}\right) + [A^-]_{aq,eq} \times \left(\frac{1-\varphi}{\varphi}\right) \quad (1.8)$$

Considering the acid dissociation constant, K_a:

$$K_a = \frac{[A^-]_{aq,eq}[H^+]_{aq,eq}}{[HA]_{aq,eq}} \quad (1.9)$$

Equation 1.8 can be rewritten as:

$$[HA]_{oil,i} = [HA]_{aq,eq} \times \left(K_p + \left(\frac{1-\varphi}{\varphi}\right) + \frac{K_a}{[H^+]_{aq,eq}} \times \left(\frac{1-\varphi}{\varphi}\right)\right) \quad (1.10)$$

For acids/bases which can dissociate, the partitioning between two immiscible phases is also expressed using the distribution coefficient, K_d, given by:

$$K_d = \frac{[HA]_{oil,eq} + [A^-]_{oil,eq}}{[HA]_{aq,eq} + [A^-]_{aq,eq}} \quad (1.11)$$

But, as for sorbic acid, the dissociation in the oil phase is practically not occurring (Wilson et al., 2000), Equation 1.11 can be rewritten as:

$$K_d = \frac{[HA]_{oil,eq}}{[HA]_{aq,eq} + [A^-]_{aq,eq}} \quad (1.12)$$

Therefore, K_p and K_d are related according to the following equation:

$$K_d = \frac{K_p}{1 + 10^{(pH-pK_a)}} \quad (1.13)$$

Inserting Equation 1.13 into 1.10, we obtain:

$$[HA]_{oil,i} = [HA]_{aq,eq} \times \left(K_d \times \left(1 + 10^{(pH-pK_a)}\right) + \left(\frac{1-\varphi}{\varphi}\right) + 10^{(pH-pK_a)} \times \left(\frac{1-\varphi}{\varphi}\right)\right) \quad (1.14)$$

By rearranging the upper, we can express $[HA]_{aq,eq}$ as a function of K_d:

$$[HA]_{aq,eq} = \frac{[HA]_{oil,i}}{(1 + 10^{(pH-pKa)}) \times \left(\left(\frac{1-\varphi}{\varphi}\right) + K_d\right)} \quad (1.15)$$

The given $[HA]_{aq,eq}$ can also be expressed as a function of K_p as follows:

$$[HA]_{aq,eq} = \frac{[HA]_{oil,i}}{\left(K_p + \left(\frac{1-\varphi}{\varphi}\right) \times (1 + 10^{(pH-pKa)})\right)} \quad (1.16)$$

$[HA]_{aq,eq}$ expressed by the Wilson model, obtained by dissolving the solute in the aqueous phase (Equation 1.4) is similar to the $[HA]_{aq,eq}$ in Equation 1.16, obtained by dissolving the solute in the oil phase. To connect one equation to the other, one needs to replace $[HA]_{oil,i}$ in Equation 1.16 with Equation 1.17 and rearrange:

$$[HA]_{oil,i} = [HA]_{aq,i} \times \left(\frac{1-\varphi}{\varphi}\right) \quad (1.17)$$

In order to validate the model, quantitative data with respect to the concentration of sorbic acid and sorbate in the aqueous and oil phase are necessary. Therefore it is not only important to calculate $[HA]_{aq,eq}$ but also the base $[A^-]_{aq,eq}$ and the undissociated acid in oil, $[HA]_{oil,eq}$. $[A^-]_{aq,eq}$ is calculated with the aid of the Henderson-Hasselbalch equation, derived from K_a:

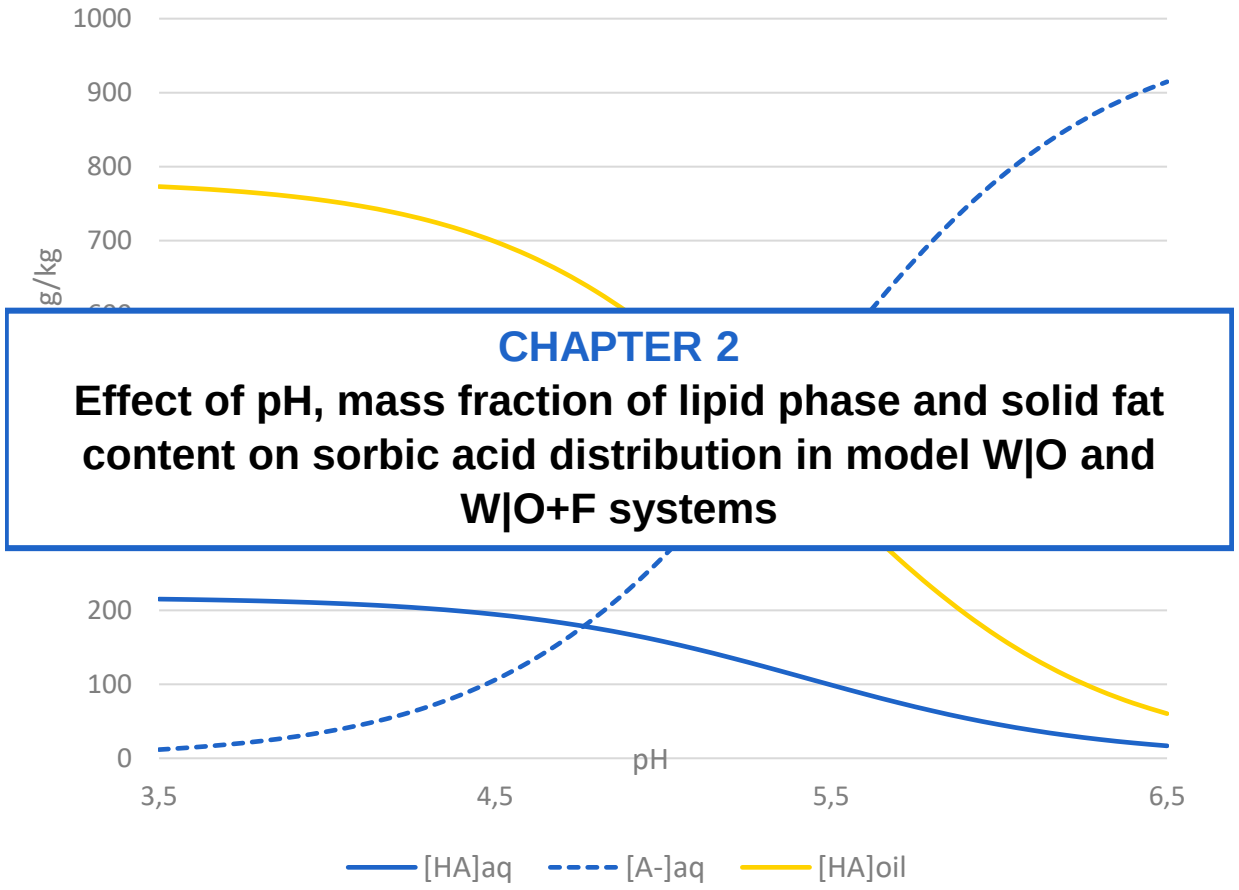
$$[A^-]_{aq,eq} = [HA]_{aq,eq} \times 10^{(pH-pK_a)} \quad (1.18)$$

$[HA]_{oil,eq}$ can then be expressed by rearranging Equation 1.7:

$$[HA]_{oil,eq} = \frac{[HA]_{oil,i} \times V_{oil} - \left([HA]_{aq,eq} + [A^-]_{aq,eq}\right) \times V_{aq}}{V_{oil}} \quad (1.19)$$

Or by considering the K_p and $[HA]_{aq,eq}$:

$$[HA]_{oil,eq} = [HA]_{aq,eq} \times K_p \quad (1.20)$$



CHAPTER 2

Effect of pH, mass fraction of lipid phase and solid fat content on sorbic acid distribution in model W|O and W|O+F systems

SUMMARY

Preservation of emulsions relies on factors including pH, temperature, structure and the addition of weak acid preservatives, such as sorbic acid. Sorbic acid tends to migrate to the lipid phase of emulsions. Taking into account the fact that undissociated sorbic acid in the aqueous phase, $(HA)_{aq,eq}$, is primarily responsible for antimicrobial activity, this partitioning behavior is considered as a loss of preservative effect. The influence of pH, mass fraction of lipid phase, and solid fat content (SFC) on the partitioning behavior of sorbic acid was investigated. A model quantifying aqueous and lipid sorbic acid concentrations in model water | oil (W|O) and water | oil+fat (W|O+F) systems was developed in Microsoft® Excel®. The predictions were validated by making model W|O and W|O+F systems where potassium sorbate was dissolved in phosphate buffers, sunflower oil was used as liquid oil and palm stearin as solid fat. Total aqueous sorbic acid, $((HA)_{aq,eq}+(A^-)_{aq,eq})$ at equilibrium was quantified by HPLC analysis. The measured values were in very good accordance with the expected values in model W|O systems. In model W|O+F systems, the deviations between the measured and expected data were slightly higher than in model W|O systems. $((HA)_{aq,eq}+(A^-)_{aq,eq})$ concentrations measured in model W|O+F systems were generally higher than those expected, especially in samples with a large SFC. This could possibly be explained by either a prolongation in the time to reach equilibrium as the fat crystals may hinder solute diffusion through the lipid phase or a change of partition coefficient (K_p) of sorbic acid due to a change in fatty acid composition when solid fat is present in the emulsion. The results indicate that the proposed model can be used as a calculation tool for concentrations of weak organic preservatives in the different phases of W|O emulsions and that control of solid fat could aid in the microbiological preservation of such emulsions.

2.1. Introduction

In W|O emulsions, only the more hydrophobic undissociated acid form migrates from the aqueous into the lipid environment. This undissociated aqueous sorbic acid form is also the form that is primarily responsible for inhibiting microbial growth (Lund et al., 2000). As microorganisms grow only in an aqueous environment, the concentration of a preservative in the aqueous phase is directly related to microbial control in a food system (Brocklehurst, 2000; Cheng et al., 2010).

Many emulsion-based formulations also contain solid fat. Typical examples in the area of food products are butter, margarine and fat spreads. In food industry, the determination of the amount of solid fat is an essential part of process control (Balinov et al., 2004).

Albeit investigations of distribution of preservatives between oil and water have been carried out (Gooding et al., 1955; Lubieniecki-von Schelhorn, 1967), a systematic investigation of the influence of pH, the mass fraction of lipid phase and the presence of solid fat on the distribution of sorbic acid in model W|O systems has not been reported.

In this study, sorbic acid was added in the form of potassium sorbate to phosphate buffers buffered at pH 3.5, 4.5, 5.5, 6.5 and allowed to partition between the aqueous and lipid phase, the latter containing either sunflower oil or a combination of sunflower oil and palm stearin. Aqueous sorbic acid concentrations were determined by HPLC and expressed as total aqueous sorbic acid, $((HA)_{aq,eq}+(A^-)_{aq,eq})$. The influence of pH, mass fraction of lipid phase in the system and solid fat content (SFC) on $((HA)_{aq,eq}+(A^-)_{aq,eq})$ in model W|O and W|O+F systems was investigated. The obtained experimental results were then compared to theoretical values calculated over the proposed preservative distribution model.

2.2. Materials and methods

2.2.1. Extension of preservative distribution model by Wilson et al. (2000) to emulsions containing liquid oil and solid fat

In order to be able to develop a correct representation of the sorbic acid distribution in W|O emulsions, it is necessary to take into account the exact volumetric mass (kg/L) and weight percentage (%) of both the lipid and aqueous phase.

Concentrations of preservatives in emulsions are generally expressed in mg/kg or ppm units. For aqueous phases in emulsions, ppm units are easily converted into mg/L or mg/kg, all three giving an equivalent result as the density of water is considered to be 1 kg/L. However, due to the lower density of the lipid phase, it is necessary to take into account that the concentration of preservative in mg/kg is not the same as in mg/L. In this case, ppm are not equivalent to mg/L.

Taking moreover into consideration that the lipid phase of emulsions consists of both liquid oil and solid fat, from Equation 1.7 we can write:

$$n_{HA_{tot}} = [HA]_{lip,eq} \times \frac{m_{lip}}{\rho_{lip}} + ([HA]_{aq,eq} + [A^-]_{aq,eq}) \times \frac{m_{aq}}{\rho_{aq}} \quad (2.1)$$

Where $n_{HA_{tot}}$ (mol) represents total moles of sorbic acid present in the entire system, $[HA]_{lip,eq}$ (mol/L) represents sorbic acid concentration in the lipid phase at equilibrium, m_{lip} (kg), ρ_{lip} (kg/L), m_{aq} (kg) and ρ_{aq} (kg/L) are the mass and volumetric mass (density) of the lipid and aqueous phase, respectively.

The solid fat content (SFC) on the total emulsion sample (%), f_{tot}, is easily measured by NMR. The SFC expressed on the lipid fraction of the sample, f_{lip}, is then given by:

$$f_{lip} = \frac{f_{tot}}{r} \quad (2.2)$$

In which *r*, the mass fraction of the lipid phase in the W|O emulsion is given by the following equation:

$$r = \frac{m_{lip}}{m_{tot}} \quad (2.3)$$

Where m_{tot} (kg) is the total mass of the W|O emulsion. Then, the liquid oil content of the lipid phase is expressed as:

$$m_{oil} = m_{lip}(1 - f_{lip}) \tag{2.4}$$

Considering Equations 2.2 and 2.4, we obtain from Equation 2.1:

$$n_{HA_{tot}} = [HA]_{lip,eq} \times \frac{m_{lip}(1 - \frac{f_{tot}}{r})}{\rho_{lip}} + ([HA]_{aq,eq} + [A^-]_{aq,eq}) \times \frac{m_{aq}}{\rho_{aq}} \tag{2.5}$$

Dividing Equation 2.5 by m_{tot}, we obtain:

$$\frac{n_{HA_{tot}}}{m_{tot}} = [HA]_{lip,eq} \times \frac{m_{lip}(1 - \frac{f_{tot}}{r})}{\rho_{lip} \times m_{tot}} + ([HA]_{aq,eq} + [A^-]_{aq,eq}) \times \frac{m_{aq}}{\rho_{aq} \times m_{tot}} \tag{2.6}$$

And inserting Equation 2.3:

$$\frac{n_{HA_{tot}}}{m_{tot}} = [HA]_{lip,eq} \times \frac{r(1 - \frac{f_{tot}}{r})}{\rho_{lip}} + \left([HA]_{aq,eq} + [A^-]_{aq,eq}\right) \times \frac{(1 - r)}{\rho_{aq}} \tag{2.7}$$

Considering Equation 1.5:

$$\frac{n_{HA_{tot}}}{m_{tot}} = K_p \times [HA]_{aq,eq} \times \frac{r(1 - \frac{f_{tot}}{r})}{\rho_{lip}} + \left([HA]_{aq,eq} + [A^-]_{aq,eq}\right) \times \frac{(1 - r)}{\rho_{aq}} \tag{2.8}$$

And considering Equation 1.18:

$$\frac{n_{HA_{tot}}}{m_{tot}} = K_p \times [HA]_{aq,eq} \times \frac{r(1 - \frac{f_{tot}}{r})}{\rho_{lip}} + \left([HA]_{aq,eq} + ([HA]_{aq,eq} \times 10^{(pH - pK_a)})\right) \times \frac{(1 - r)}{\rho_{aq}} \tag{2.9}$$

Extracting the $[HA]_{aq,eq}$ terms:

$$\frac{n_{HA_{tot}}}{m_{tot}} = [HA]_{aq,eq} \times \left(K_p \times \frac{r(1 - \frac{f_{tot}}{r})}{\rho_{lip}}\right) + [HA]_{aq,eq} \times (1 + 10^{(pH - pKa)}) \times \frac{(1 - r)}{\rho_{aq}} \tag{2.10}$$

Finally, we rearrange Equation 2.10 as a function of $[HA]_{aq,eq}$ to obtain the final equation form:

$$[HA]_{aq,eq} = \frac{n_{HA_{tot}}}{m_{tot}\left(K_p \times \frac{r(1 - \frac{f_{tot}}{r})}{\rho_{lip}} + (1 + 10^{(pH - pKa)}) \times \frac{(1 - r)}{\rho_{aq}}\right)} \tag{2.11}$$

In Equation 2.11, $[HA]_{aq,eq}$ is expressed on a mole to volume basis (mol/L). A common practice in the industry is to express additive concentrations in weight (mg/kg or ppm) units. In order to convert $[HA]_{aq,eq}$ to a mg/kg basis, the molecular weight of sorbic acid should be considered.

In the case of the $[HA]_{lip,eq}$ term, both the molecular weight of sorbic acid and the density of the lipid phase should be considered when converting mol/L to mg/kg. .

To differentiate the sorbic acid concentrations expressed on a mol/L basis from the same concentrations on mg/kg basis, a different notation is introduced. $(HA)_{aq,eq}$, $(A^-)_{aq,eq}$ and $(HA)_{lip,eq}$ represent the sorbic acid concentrations in the individual phases of W|O emulsions on a weight (mg/kg) basis. The relationship between the terms is given by:

$$(HA)_{aq,eq} = \frac{[HA]_{aq,eq} \times MW_{HA}}{\rho_{aq}} \quad (2.12)$$

Where MW_{HA} is 112.13 g/mol and ρ_{aq} is ca. 1000 g/L.

$$(A^-)_{aq,eq} = \frac{[A^-]_{aq,eq} \times (MW_{HA} - MW_{H^+})}{\rho_{aq}} \quad (2.13)$$

Where MW_{H+} is the molecular weight of a hydrogen ion, ca. 1 g/mol.

$$(HA)_{lip,eq} = \frac{[HA]_{lip,eq} \times MW_{HA}}{\rho_{lip}} \quad (2.14)$$

Where ρ_{lip} for sunflower oil is ca. 920 g/L at 25 °C (Sigma Aldrich, 2019).

2.2.2. Experimental validation of sorbic acid distribution model

2.2.2.1. Preparation of aqueous phase of model W|O and W|O+F systems

The aqueous phases of W|O and W|O+F systems were prepared according to European Pharmacopoeia 7.0. (Council of Europe, 2004). Phosphate buffers were prepared and buffered at pH 3.5, 4.5, 5.5, 6.5, mimicking the pH range of oil and fat based products (Andersen and Williams, 1954; Roberts et al., 2005).

Phosphate buffer at pH 3.5 was prepared by dissolving 6.8 g of KH_2PO_4 in 1 L of distilled water. The original protocol demanded for 68 g of KH_2PO_4 in 1 L of distilled water, but this elevated concentration of the phosphate salt later resulted in formation of a crystalline substance in the HPLC vials due to solubility limits of potassium dihydrogen phosphate in ethanol. Thus, the protocol for pH 4.5 was used and the pH was poised to 3.5. Phosphate buffer at pH 4.5 was prepared by dissolving 6.8 g of KH_2PO_4 in 1 L of distilled water. Phosphate buffer at pH 5.5 was prepared by dissolving 13.61 g of KH_2PO_4 in 1 L of distilled water (solution A). Then, 35.81 g of Na_2HPO_4 was dissolved in 1 L of distilled water (solution B). 96.4 mL of solution A was

mixed with 3.6 mL of solution B. Phosphate buffer at pH 6.5 was prepared by dissolving 13.8 g of $NaH_2PO_4 \times H_2O$ in 1 L of distilled water. The pH's of the buffers were poised using 6 M H_3PO_4 or 10 M NaOH, depending if the pH of the solution respectively needed to be acidified or alkalified. Potassium sorbate was dissolved in each of the aqueous phases at a concentration of 1340 mg/L aqueous phase, equivalent to 1000 mg/L of sorbic acid. pH was then readjusted in buffers at pH 3.5 and 4.5, because adding sorbate would slightly elevate the pH. All reagents were obtained from Merck (Germany).

2.2.2.2. Preparation of lipid phase of model W|O and W|O+F systems

In the case of model W|O systems, sunflower oil (Vandemoortele NV) was used as lipid phase. In the case of model W|O+F systems, sunflower oil and palm stearin (Vandemoortele NV) were mixed in varying ratios. Palm stearin was first melted in a 1 L glass beaker by microwaving (duration varying depending on sample weight) and then lipid mixtures containing 35, 70, 100% of palm stearin with sunflower oil were prepared in 2 L glass jars with a lid. The lipid mixtures were homogenized with Ultra Turrax (IKA Werken, Germany) at 10000 rpm for 5 minutes, and allowed to cool down to 44 °C.

2.2.2.3. Preparation of model W|O and W|O+F systems

MODEL W|O SYSTEMS

When investigating the influence of pH on the distribution of sorbic acid, 100 mL of phosphate buffer with 1000 mg/L sorbic acid, as described in Paragraph 2.2.2.1. was poured in a 500 mL Schott bottle. Sunflower oil, 100 g, was poured over the aqueous phase. The model W|O system was shaken for 4 hours at 200 rpm (IKA Werken). Partitioning of sorbic acid was performed by using the classic "shake-flask method", as described in Berthod and Broch (2004). Model W|O systems were then let to equilibrate for 1 week at 22 °C and 7 °C. Afterwards, they were poured into separatory funnels, for ease of sampling and the aqueous phase was sampled for sorbic acid.

In the case of investigating the influence of the mass fraction of lipid phase, *r*, on the distribution of sorbic acid, varying amounts of phosphate buffer at pH 3.5, 4.5, 5.5, 6.5 and sunflower oil were brought together. The total mass of the model W|O system was 200 g, with 20, 40, 60, 80% of sunflower oil by weight in each of the systems. The model W|O systems with varying mass fractions of lipid phase in the system were shaken for 4 hours at 200 rpm and sampled after 1 week, as described above.

MODEL W|O+F SYSTEMS

When investigating the influence of solid fat content on the distribution of sorbic acid, both the aqueous and lipid phase were kept at 44 °C before combining. The reasons were twofold: (i) the lipid phase is still liquid and easy to handle at this temperature, (ii) when the aqueous phase was colder than the lipid phase, pouring the warm lipid phase on top of the aqueous phase created an uneven interface. First, 100 mL of phosphate buffers at each pH were poured in 500 mL Schott bottles. Then, 100 g of lipid mixtures as described in Paragraph 2.2.2.2. were added on top of the aqueous phase. These samples were not shaken, due to the solidification of lipid phase, but were let to stand still and equilibrate for 8 weeks.

All the samples of the three model systems described above, designed to investigate (i) the influence of pH, (ii) the influence of mass fraction of lipid phase, *r*, and (iii) the influence of SFC on the distribution of sorbic acid in model W|O and W|O+F systems, were made in triplicate. Systems (i) and (ii) were made on 3 separate days, from 3 separately prepared phosphate buffer stock solutions and the concentrations of sorbic acid were analyzed after 1 week. In the case of (iii), it was decided to follow the sorbic acid partitioning during time, thus model W|O+F systems were prepared and sampled at week 1, 2, 3, 6, 8.

A schematic representation of the model W|O and W|O+F systems is shown on Figure 2.1.

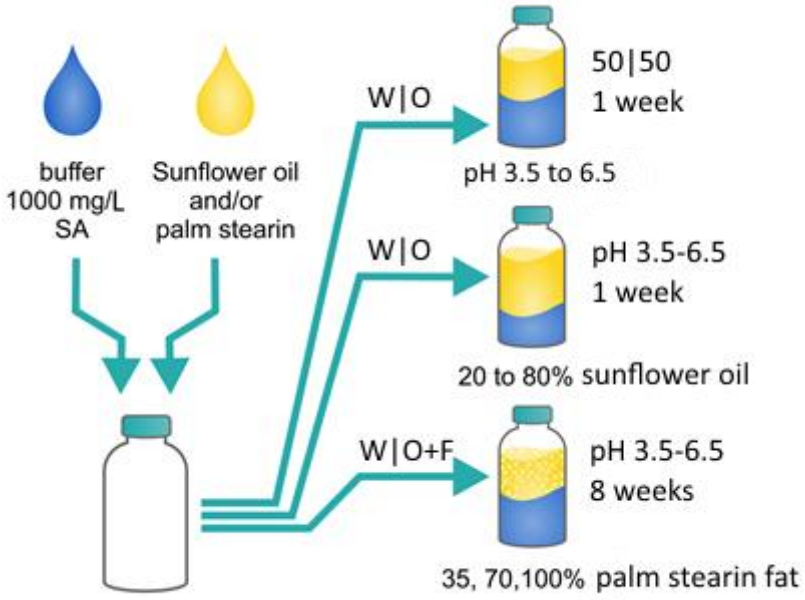


Figure 2.1. Schematic representation of model W|O and W|O+F systems used in experiments investigating the distribution of sorbic acid in biphasic systems. SA represents sorbic acid.

2.2.2.4. HPLC analysis of sorbic acid in model W|O and W|O+F systems

From the aqueous phase of the food model emulsion, 50 µL was sampled and brought in an HPLC vial (Agilent Technologies, USA) with 450 µL of an extraction solvent composed of 70% of ethanol (Milipore, USA) plus 30% of HPLC grade distilled water (VWR, USA) and 500 µL of 100 mg/L vanillin solution (Merck, USA) used as internal standard. The analysis was performed as described by Pylypiw and Grether (2000) using HPLC with a Waters chromatograph (Waters, USA) equipped with an Alliance e2695 isocratic pump and a Waters 2487 UV detector set at 280 nm. 10 µL of sample was injected into the column. The sample was eluted isocratically with a solution of HPLC water/acetonitrile/acetic acid (74/26/0.5 v/v/v) used as the mobile phase through a Merck LiChroCART® 250-4 LiChrospher 100 reversed phase C_{18} column (25 cm x 4 mm, Merck, USA) at a rate of 0.9 mL/min and oven temperature set at 30 °C. The software used for the collection and the processing of data was Empower v.3. A solution of 100 mg/L of sorbic acid (Sigma Aldrich) in the extraction solvent described above was used as reference. Injection was performed in duplicate.

2.2.2.5. Solid fat content (SFC) measurements

SFC was measured using a Maran Ultra 23 MHz pulsed Nuclear Magnetic Resonance (pNMR) (Oxford Instruments, UK). The SFC was measured from the stock fat mixtures kept in 2 L closed glass jars kept at room temperature and dark place after 8 weeks of stand still. NMR-tubes were filled with the lipid mixtures kept at 22 °C. Using calibration standards (0.0, 29.3, 70.5% SFC), SFC values were derived. SFC measurements of the lipid phases revealed an average 13.6, 29.7, 60.6% SFC (f_{lip}) in model W|O+F systems with 35, 70, 100% palm stearin used as lipid phase, respectively. Both the sample preparation and the NMR analyses were performed in triplicate.

2.2.2.6. Statistical analysis

Minitab® 17.1.0 was used for basic data exploration. Normality was tested with the Anderson-Darling Normality test. Homoscedasticity was tested with F-tests. The means of total aqueous sorbic acid concentrations ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) / (mg/kg)) were compared by 2-sample t-tests. A 5% significance level was applied for all statistical tests.

2.3. Results and discussion

2.3.1. Theoretical development of sorbic acid distribution model in W|O emulsions

A Microsoft®Excel® template was developed to calculate undissociated sorbic acid and conjugated base concentrations in the aqueous phase, $(HA)_{aq,eq}$ and $(A^-)_{aq,eq}$ respectively and undissociated acid concentrations in the lipid phase of a W|O emulsion, $(HA)_{lip,eq}$, as well as to visualize the behavior of sorbic acid across varying pH and *r* ranges.

This template can be used for any weak monoprotic organic acid, such as sorbic and benzoic acid, used to preserve a W|O emulsion. The system characteristics were entered, i.e. the molecular weight (112.13 g/mol), K_p (3.3) and pK_a (4.75) of sorbic acid, as well as the density (volumetric mass) of both the aqueous (1000 g/L) and lipid phase (920 g/L). Then, the conditions, i.e. total mass (kg), mass fraction of lipid phase, *r,* the final pH of the product, measured SFC (on total W|O emulsion, f_{tot}) and total mass of potassium sorbate or sorbic acid (kg) were entered, in order to calculate the theoretical concentrations of (i) $(HA)_{aq,eq}$, (ii) $A^-)_{aq,eq}$, and (iii) $(HA)_{lip,eq}$ at equilibrium.

It is necessary to mention that the K_p value used in the calculations is dimensionless, but represents the quotient of molar concentrations (mol/L) of sorbic acid in the lipid and aqueous phase (see Chapter 1). In industry, it is also common to work in mass concentrations. By expressing the concentration terms in mass, the K_p is underestimated because one liter of sunflower oil doesn't correspond to one kilogram but to ca. 0.92 kilograms of sunflower oil (Sigma Aldrich, 2019). In this case, the molar based K_p (3.3) is divided by the density of sunflower oil (0.92 kg/L) to obtain a mass based K_p (3.59).

Figure 2.2 shows the development of the three concentrations mentioned above as a function of pH. In the calculation template the default output is in ppm units (mg/kg). The conditions used to plot Figure 2.2 were the same conditions used for experimental validation. The mass fraction of the lipid phase was 0.5, total sorbic acid concentration in the model W|O system was 500 mg/kg (i.e. the initial 1000 mg/L concentration in the aqueous phase divided between 50% of aqueous and 50% of lipid phase) and the pH range was between 3.5 and 6.5.

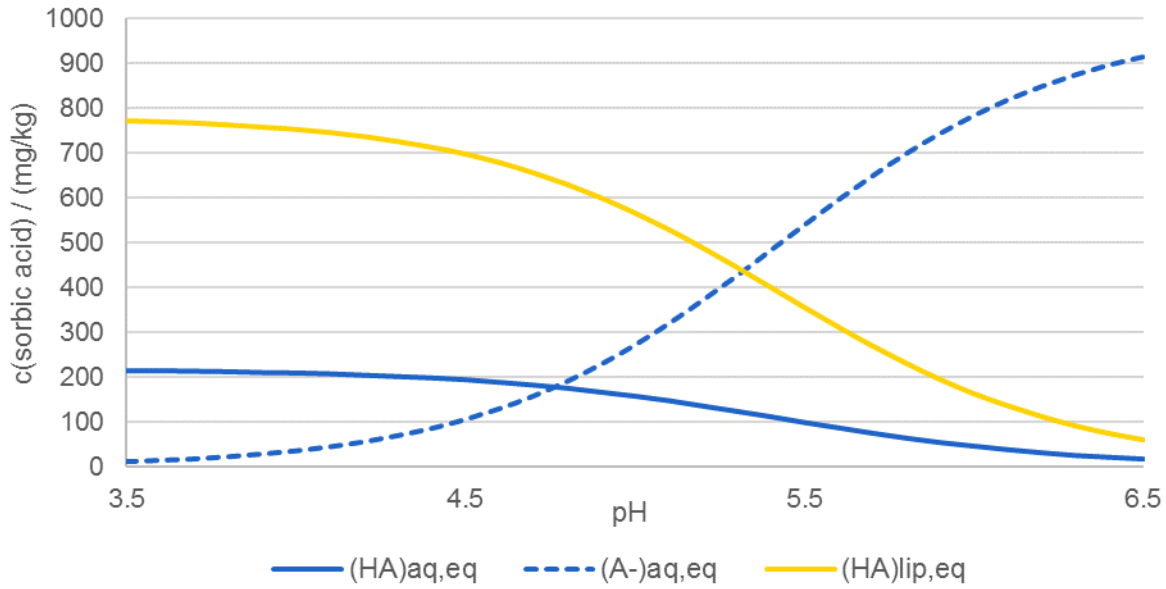


Figure 2.2. Theoretical development of sorbic acid behavior in model W|O systems as a function of pH. $(HA)_{aq,eq}$ is the concentration of undissociated acid (i.e. inhibitory form) in the aqueous phase, $(A^{-})_{aq,eq}$ is the concentration of the dissociated acid in the aqueous phase and $(HA)_{lip,eq}$ is the undissociated acid in the lipid phase.

At pH 3.5, almost all of the sorbic acid is present in its undissociated form (95% of total added sorbic acid), due to this pH being lower than the pK_a of sorbic acid. In this case most of the undissociated acid has partitioned in the lipid phase, rendering this as a loss of the preservative. Nevertheless, the acid remaining in the aqueous phase is mostly present in its microbiologically inhibitory form, $(HA)_{aq,eq}$. Increasing pH in the system gives way to formation of the dissociated form. Due to $(A^{-})_{aq,eq}$ being a polar moiety, it is not able to partition into the non-polar lipid phase. Effectively, this means that at a relatively high pH, like 6.5, almost all the acid will remain in the aqueous phase, but it will exert little inhibitory activity, although Eklund (1983) claimed that the dissociated sorbic acid form also exhibits inhibitory activity, 10-600 times less than the undissociated form.

Figure 2.3 depicts $(HA)_{aq,eq}$ in model W|O systems as a function of the mass fraction of lipid phase, *r*. The conditions used to plot Figure 2.3 are the same as those ones mentioned in Figure 2.1, other than the mass fraction of the lipid phase, which varied from 0.1 to 0.9 by weight. At pH 3.5, the majority of sorbic acid present in the system is undissociated, thus able to partition into the lipid phase. As *r* increases, $(HA)_{aq,eq}$ decreases, due to the decreased aqueous and increased lipid volume being available for the $(HA)_{aq,eq}$ to partition into. Similar behavior is observed at pH 4.5. Interestingly, at pH 5.173, an increase in *r* doesn't cause a change in $(HA)_{aq,eq}$. Below this value, at a given pH, a higher fraction of lipid phase corresponds to a lower sorbic acid concentration in the aqueous phase. Above this pH, a higher fraction of lipid phase aids to increase $(A^{-})_{aq,eq}$. Another interesting phenomenon is the slight increase of

$(HA)_{aq,eq}$ at pH 6.5 at an increasing *r*. This is because almost all of the sorbic acid is present in its deprotonated form in a relatively small volume of aqueous phase. This increase in $(A^-)_{aq,eq}$ causes a proportional, but, in practical implications, effectively negligible increase in $(HA)_{aq,eq}$.

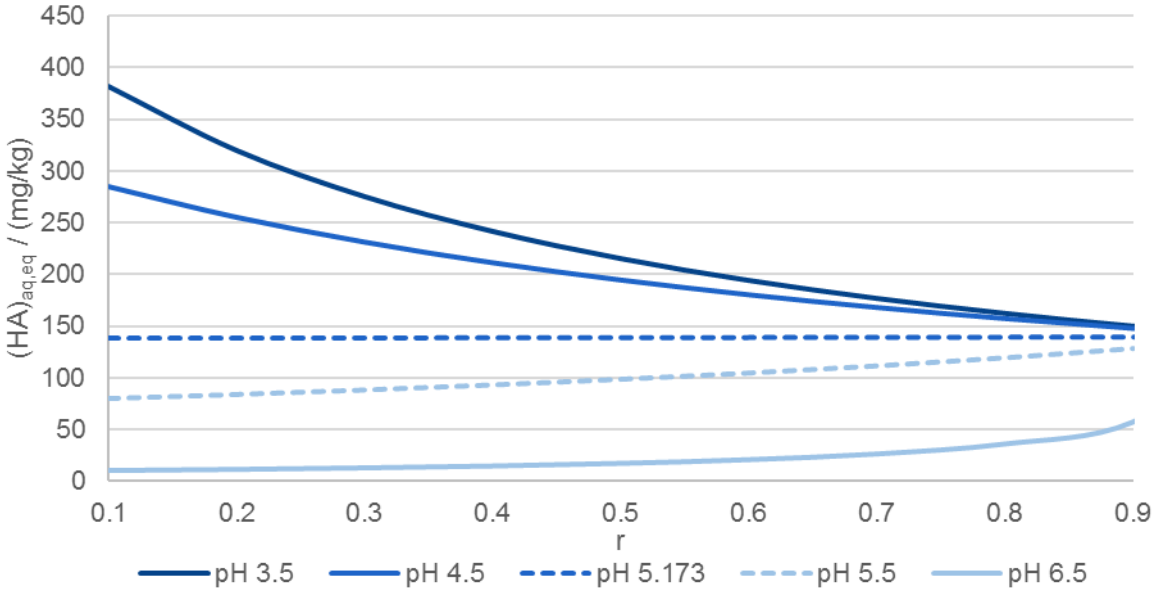


Figure 2.3 Theoretical development of $(HA)_{aq,eq}$ behavior in model W|O systems as a function of the mass fraction of the lipid phase, *r* (i.e. 0.60 is equivalent to 60% mass fraction of lipid phase).

The developed Microsoft®Excel® template can also be used to calculate the necessary mass of sorbic acid to be added into a W | O emulsion, to obtain a desired $(HA)_{aq,eq}$ concentration. At lower pH levels it is feasible to add an amount of sorbic acid that will remain under the EU legal limit (i.e. 1000 and 2000 mg/kg on total mass for emulsions with more and less than 60% fat, respectively) and still achieve a relatively high inhibitory $(HA)_{aq,eq}$. At pH 6.5, where most acid is dissociated, the lower the *r*, the higher the mass of sorbic acid necessary to obtain a certain active concentration. This is why, in case of the W | O emulsion having a high pH, it is easy to breach the maximum allowed sorbic acid limit. Thus, in W | O emulsions with a high pH (6.0-6.5), preservation is primarily achieved by adding other preservatives such as salt, but also by manipulating the droplet size of the discontinuous phase and temperature control (Prachaiyo and Mclandsborough, 2006).

When theoretically assessing the influence of solid fat content on the distribution of sorbic acid in model W|O+F systems, we assumed that solid fat is inert, meaning that sorbic acid will not be able to dissolve in the fat crystals, but only in the liquid part of the lipid phase. Figure 2.4 shows the theoretical development of sorbic acid behavior in model W|O+F systems as influenced by f_{tot} (SFC). The above mentioned figure was plotted with an initial sorbic acid concentration of 1000 mg/L of sorbic acid in the aqueous phase and the system was comprised out of 50% aqueous and 50% lipid phase.

According to Figure 2.4, in the case of the inhibitory sorbic acid concentration, $(HA)_{aq,eq}$, at a low pH (e.g. 3.5), where most of the sorbic acid is protonated and able to partition into the lipid phase, the higher the f_{tot} in the system, the higher the $(HA)_{aq,eq}$ concentration. At higher pH values (e.g. 5.5 and 6.5), an increase in f_{tot} doesn't exceedingly contribute to the change in $(HA)_{aq,eq}$ due to the small amount of the undissociated form of sorbic acid initially being present in the system. A similar trend is observed for total aqueous sorbic acid, $((HA)_{aq,eq}+(A^-)_{aq,eq})$, which changes proportionally with the change in SFC. This is again best seen when observing the influence of SFC on $((HA)_{aq,eq}+(A^-)_{aq,eq})$ at pH 3.5. The crystalline lipid phase might serve as a barrier, preventing the preservative to diffuse out of the aqueous phase.

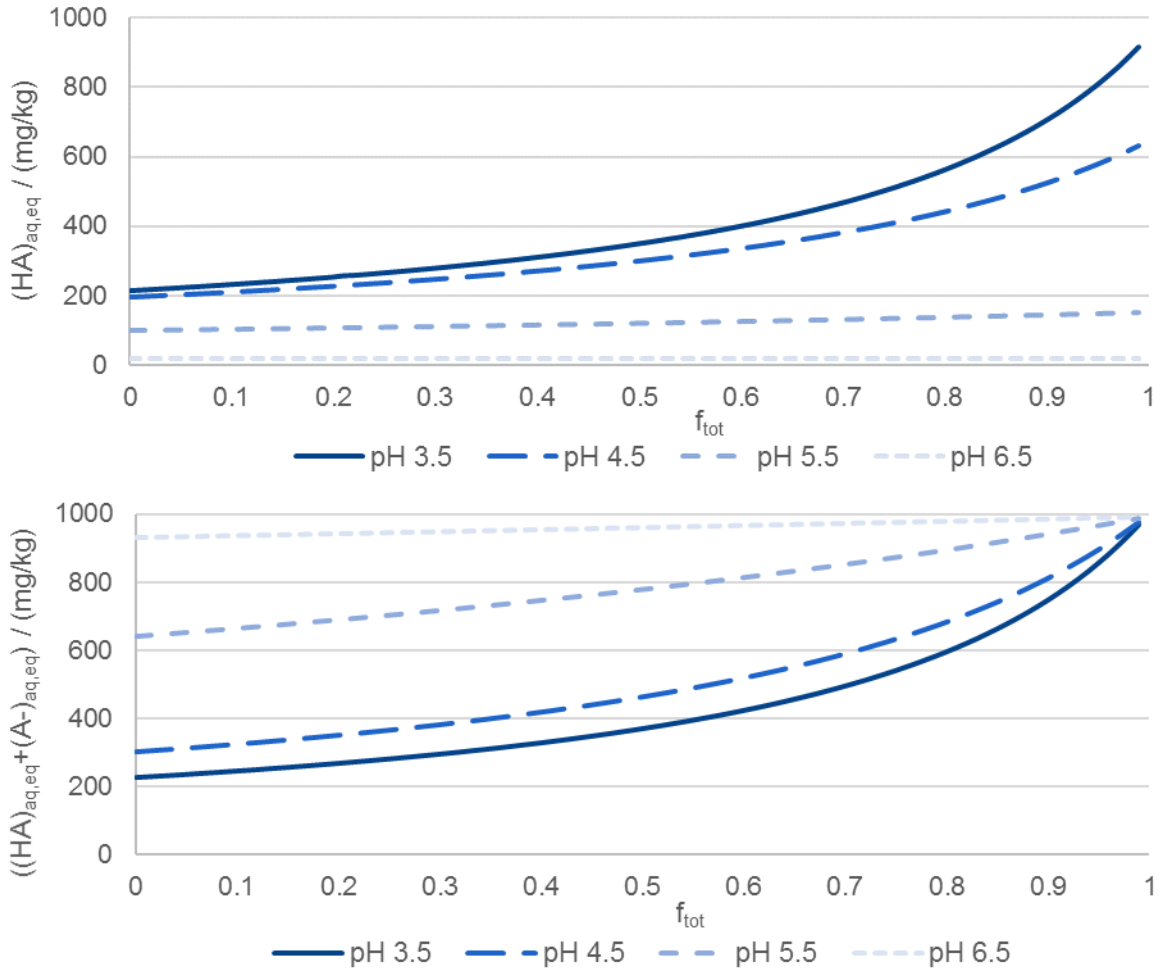


Figure 2.4 Theoretical development of sorbic acid behavior in model W|O+F systems as a function of f_{tot}. The upper image represents the development of the active inhibitory form of sorbic acid, $(HA)_{aq,eq}$ while the lower image represents total aqueous sorbic acid, $((HA)_{aq,eq}+(A^-)_{aq,eq})$.

2.3.2. Experimental validation of sorbic acid distribution model: effect of pH on $(HA)_{aq,eq}$

Figure 2.5 shows the expected versus measured $((HA)_{aq,eq}+(A^-)_{aq,eq})$ in model W|O systems at 22 °C prepared as described in Paragraph 2.2.2.3. The measured values represent $((HA)_{aq,eq}+(A^-)_{aq,eq})$ concentrations analyzed by HPLC. The expected values are $((HA)_{aq,eq}+(A^-)_{aq,eq})$ calculated over the theoretical model described in Paragraph 2.2.1. It is necessary to mention that the final pH (after 4 hours shaking at 220 rpm and 1 week stand still) of the aqueous phases of model W|O systems increased (initial values of pH 3.5, 4.5, 5.5, 6.5). This was due to the sorbic acid partitioning phenomenon. E.g. at pH 3.5, the added sorbic acid is mostly present in its undissociated form, which preferably partitions into the lipid phase. As the undissociated acid partitions, the final pH of the aqueous phase also increases. The expected $((HA)_{aq,eq}+(A^-)_{aq,eq})$ were calculated according to the measured final pH values. Final pH values of the aqueous phases of all experimental conditions are listed in Table 2.1.

Table 2.1. Measured pH values in aqueous phases of model W|O systems at 22 °C and 7 °C. Average and standard deviation are calculated from 9 replicates.

Temperature	pH 3.5	pH 4.5	pH 5.5	pH 6.5
22 °C	3.68 ± 0.01	5.08 ± 0.03	5.70 ± 0.01	6.56 ± 0.03
7 °C	3.66 ± 0.05	5.04 ± 0.05	5.70 ± 0.01	6.57 ± 0.01

The measured concentrations at 22 °C showed very strong correspondence to the proposed model, although some discrepancies were observed for samples at pH 4.5. These discrepancies were observed systematically at pH 4.5, probably due to the weak buffering capacity of the prepared phosphate buffer at the above mentioned pH. Nevertheless, when taking into account all the measured $((HA)_{aq,eq}+(A^-)_{aq,eq})$ concentrations at all pH levels, it was evident that pH was key in explaining the variability of the $((HA)_{aq,eq}+(A^-)_{aq,eq})$ concentration in the aqueous phase of a model W|O system. According to experimental results, our proposed model was able to predict $((HA)_{aq,eq}+(A^-)_{aq,eq})$ in a 50|50 W|O emulsion in a pH range of 3.5 to 6.5.

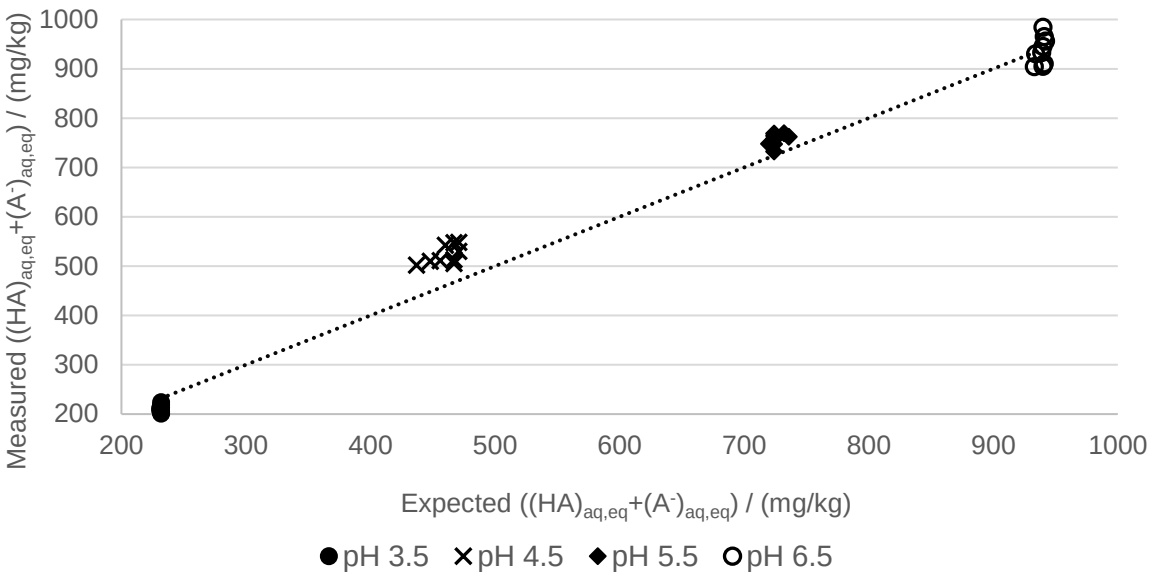


Figure 2.5 Expected versus measured ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) in model W|O systems at 22 °C. Replicates were prepared on three separate days in triplicates. HPLC injection performed in duplicate. The dotted trend line represents an ideal scenario, where expected values are equal to the measured values. BF=0.98. AF=1.08.

Considering that food emulsions can be kept at both room and refrigeration temperature, it was decided that it was necessary to test for the influence of temperature on $((HA)_{aq,eq}+(A^-)_{aq,eq})$ in model W|O systems. Namely, temperature affects the partition coefficient, K_p, of an organic solute between two solvent phases (Chiou, 2003). Determining exact temperature dependence of physico-chemical properties of a solute is an important factor for improving the predictive capability of environmental models (Finizio and Di Guardo, 2001). The measured concentrations at 7 °C (Figure 2.6) showed strong correspondence to the proposed distribution model, with the same slight discrepancies between expected and measured $((HA)_{aq,eq}+(A^-)_{aq,eq})$ being observed for samples at pH 4.5, as seen at 22 °C. After homogeneity of variance was confirmed, 2-sample t-tests were performed at each pH to assess if the average $((HA)_{aq,eq}+(A^-)_{aq,eq})$ were the same at 7 °C and 22 °C. Average $((HA)_{aq,eq}+(A^-)_{aq,eq})$ concentrations at pH 3.5, 4.5, 5.5, 6.5 were not significantly different ($p>0.05$) between samples kept at 22 °C and 7 °C, indicating that, in our case, temperature had no effect on the distribution properties of sorbic acid at equilibrium.

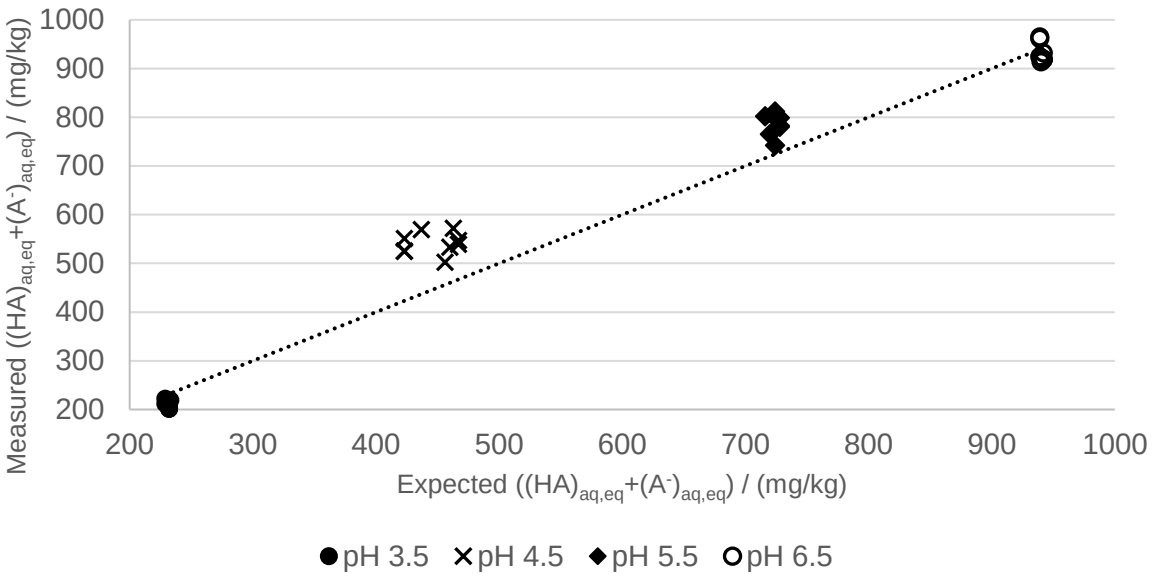


Figure 2.6 Expected versus measured ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) in model W|O systems at 7 °C. Replicates were prepared on three separate days in triplicate. HPLC injection performed in duplicate. The dotted trend line corresponds to an ideal situation, where expected values are equal to the measured values. BF = 0.97. AF = 1.11.

Before a model can be used in practice, it needs to show that it accurately predicts the behavior of the components in a system. The demonstration of this ability is called "validation". When considering model performance, it is of most interest whether the model produces predictions that will overestimate the concentration of the inhibitory form, $(HA)_{aq,eq}$. This, in turn, might increase the chance of inadequate preservative dosage during product formulation and subsequently the actual active preservative concentration might be too low to ensure a safe and quality product. To assess the performance of the proposed model, an approach postulated by Ross (1996) was used. Two factors that were proposed were the Bias factor (BF) (Equation 2.15) and the Accuracy factor (AF) (Equation 2.16).

$$Bias\ factor\ (BF) = 10^{\left(\sum \log((HA)_{aq,eq_{exp}}/(HA)_{aq,eq_{meas}})/n\right)} \tag{2.15}$$

$$Accuracy\ factor\ (AF) = 10^{\left(\sum \log\left|((HA)_{aq,eq_{exp}}/(HA)_{aq,eq_{meas}})\right|/n\right)} \tag{2.16}$$

Where $(HA)_{aq,eq_{exp}}$ denotes the expected sorbic acid concentrations predicted by the model, $(HA)_{aq,eq_{meas}}$ denotes the measured aqueous sorbic acid concentrations, and *n* is the number of observations.

The BF and AF can be interpreted as quantitative summaries of the plot used to evaluate the performance of a kinetic model. The BF answers the question whether, on average, the measured values lie above or below the line of equivalence (i.e. the bisectrix – where expected values are equal to the measured), and, if so, by how much. Thus it assesses whether the model is generally “fail-safe” or “fail dangerous”. The accuracy factor averages the minimum distance between each point and the line of equivalence. It is, thus, a measure of average deviation and may be used as a simple indicator of the level of confidence one may have in the model’s predictions.

Complete agreement between predictions and measured values will lead to a BF of 1. A BF of 1.1 indicates that not only the model is “fail-dangerous” because it predicts bigger values than are measured, but it indicates that the predictions exceed the measured values on average by 10%. Under- and over-prediction will tend to ‘cancel out’ in the BF because the logarithm of the $(HA)_{aq,eq}$ ratios will have opposite signs. Thus, another measure is introduced, the AF, which is the antilogarithm of the average of absolute logarithmic ratio values. This value will always be greater than or equal to 1, and the larger the value, the less accurate is the average estimate.

In the case of the data represented by Figure 2.5 (situation at 22°C), the BF was 0.98 and the AF was 1.08. The BF value indicates that the model predicted lower values than those measured, by 2% in average. Thus, it indicates that the model is “fail-safe”, i.e. that the true aqueous sorbic acid concentrations are slightly higher than what the model predicts. Provided that the BF is close to 1, the AF is almost equivalent to the 50% confidence interval calculated from the standard deviation of the concentration ratios. Considering the AF for the same data, the predictions are, on average within 8% of the observation. Thus, for an expected sorbic acid concentration of 100 mg/kg in a model W|O system at 22 °C, the bounds expected to encompass approximately half of the observations would be 93-108 mg/kg (i.e. 100/1.08 and 100×1.08).

In the case of the model W|O system at 7 °C (Figure 2.6), the BF was 0.97 and the AF was 1.11. This indicates that the model predicted lower values than measured, by 3% on average (fail-safe model). For an expected sorbic acid concentration of 100 mg/kg, the bounds expected to encompass approximately half of the observations would be 90-111 mg/kg.

2.3.3. Experimental validation of sorbic acid distribution model: effect of *r* on $(HA)_{aq,eq}$

Figure 2.7 depicts the HPLC results of $((HA)_{aq,eq}+(A^-)_{aq,eq})$ in a model W|O system at pH 3.5, 4.5, 5.5, 6.5 at varying mass fractions of lipid phase, *r*, where 0.2, 0.4, 0.6, 0.8 signifies model W|O systems with 20, 40, 60, 80% of lipid phase by weight, respectively. As was the case with the model W|O systems described in Paragraph 2.3.2., the final pH values measured after 4 hours of shaking at 220 rpm and 1 week, were, in general, higher than the initial pH values. The averages and standard deviations of final pH values measured in all the systems are listed in Table 2.2. The expected $((HA)_{aq,eq}+(A^-)_{aq,eq})$ were calculated by the model proposed in Paragraph 2.2.1, at final pH values stated in Table 2.2.

It can be seen from Figure 2.7 that, in model W|O systems at pH 3.5, 4.5, 5.5, 6.5, the mass fraction of lipid phase is a good predictor of $((HA)_{aq,eq}+(A^-)_{aq,eq})$. The BF calculated for this system was 0.98 and the AF 1.08.

The literature on the influence of varying ratios of water to oil on the distribution of preservatives in W|O emulsions is scarce. Wilson et al. (2000) validated their proposed organic acid distribution model by investigating the influence of adding varying volumes of sunflower oil on the final pH in TSBYG (microbiological growth media)|sunflower oil systems. The pH of TSBYG was poised to 6.0 and 0.01 mol/L of sorbic acid was added. The volumes of sunflower oil were 0, 20, 50, 70, 80, 90, 100% of total volume. They claimed that their proposed distribution model was a convenient and reliable method for predicting the effect of weak acids on the pH of complex buffering system. This was proven by measuring the pH of the varying TSBYG|sunflower oil systems, which corresponded to the expected values. However, exact aqueous sorbic acid concentrations hadn't been elucidated in the publication. In our study, the $((HA)_{aq,eq}+(A^-)_{aq,eq})$ were quantified and show good correspondence to the concentrations predicted by the proposed preservative distribution model.

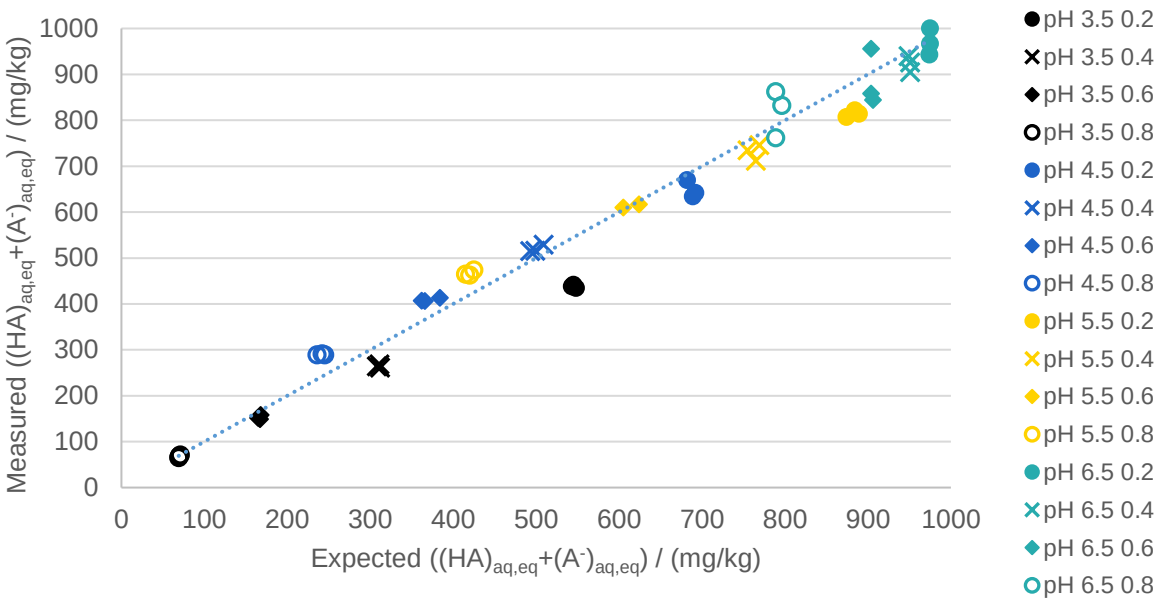


Figure 2.7 Expected versus measured ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) in model W|O systems with varying mass fractions of lipid phase, *r*, at 22 °C. Model W|O systems of varying pH were prepared with 20, 40, 60, 80% lipid phase by mass (i.e. r = 0.2, 0.4, 0.6, 0.8, respectively) on three separate days. HPLC injection performed in duplicate. The dotted trend line corresponds to an ideal situation, where measured values are equal to the expected values. BF = 0.98. AF=1.08.

Table 2.2. Measured pH values in aqueous phases of model W|O systems of varying mass fractions of lipid phase, *r*.

r	pH 3.5	pH 4.5	pH 5.5	pH 6.5
0.2	3.65 ± 0.06	4.76 ± 0.02	5.55 ± 0.04	6.48 ± 0.02
0.4	3.66 ± 0.06	4.91 ± 0.03	5.60 ± 0.02	6.49 ± 0.01
0.6	3.66 ± 0.06	5.10 ± 0.03	5.66 ± 0.03	6.50 ± 0.01
0.8	3.66 ± 0.06	5.32 ± 0.02	5.74 ± 0.01	6.51 ± 0.01

2.3.4. Experimental validation of sorbic acid distribution model: effect of f_{tot} on $(HA)_{aq,eq}$

Figure 2.8 shows the $((HA)_{aq,eq}+(A^-)_{aq,eq})$ in model W|O+F systems during time. As mentioned in Paragraph 2.2.2.3., the trial period was 8 weeks, with several sampling times in between, to follow the time dependency of the partitioning of sorbic acid. The $((HA)_{aq,eq}+(A^-)_{aq,eq})$ decreased during 8 weeks, showing the time dependent partitioning of sorbic acid. This implies that the sorbic acid diffuses out of the aqueous phase more slowly in emulsions with solid fat than in emulsions with only liquid oil. Practically, this could mean that, for microorganisms residing in the aqueous phase, the time of exposure to sorbic acid would be prolonged, in turn increasing the effectiveness of the preservative.

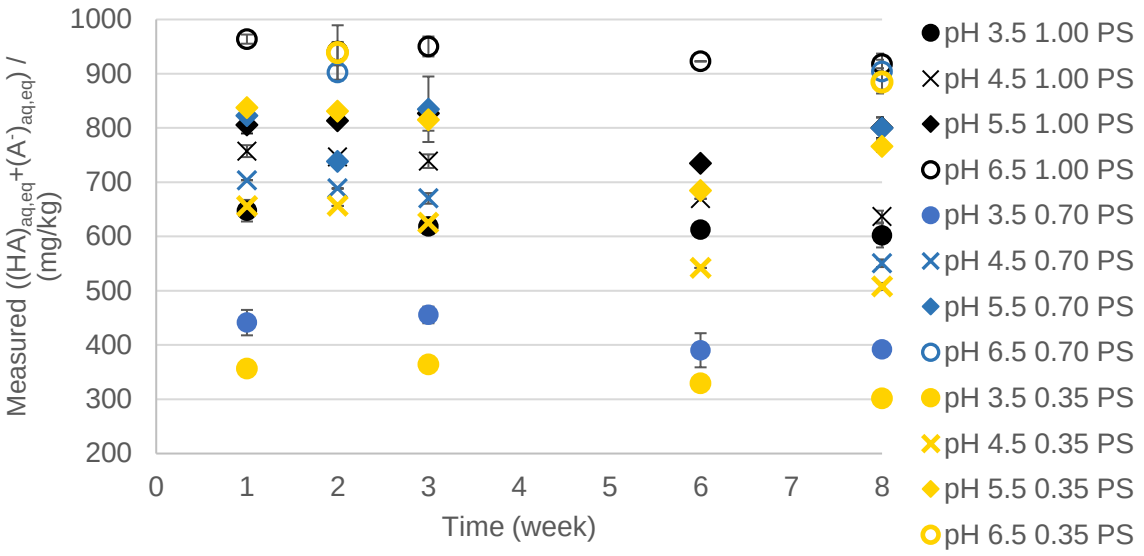


Figure 2.8 Measured ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) in model W|O+F systems of varying f_{tot} during time. 1.00, 0.70, 0.35 PS denotes model emulsions with 100, 70, 35% palm stearin, respectively. Error bars denote standard deviation.

It was not possible to sample from the same recipient, because to reach the aqueous phase, the solidified lipid phase had to be destroyed. However, replicates of all the model W|O+F systems were made from the same buffer stock solution and stock lipid phases, improving result certainty. As was the case in Paragraph 2.3.2. and 2.3.3., the pH values after 8 weeks differed from the initial pH values. Table 2.4 lists the pH changes of the model W|O+F systems.

Table 2.4. Measured pH values of aqueous phases of model W|O+F systems with varying f_{tot} after 8 weeks. 1.00, 0.70, 0.35 PS denotes model emulsions with 100, 70, 35% palm stearin, respectively. Average and standard deviation are calculated from three replicates.

System	pH 3.5	pH 4.5	pH 5.5	pH 6.5
1.00 PS	3.46 ± 0.01	4.71 ± 0.01	5.56 ± 0.01	6.48 ± 0.01
0.70 PS	3.51 ± 0.02	4.61 ± 0.01	5.54 ± 0.01	6.49 ± 0.00
0.35 PS	3.51 ± 0.00	4.76 ± 0.03	5.59 ± 0.01	6.50 ± 0.00

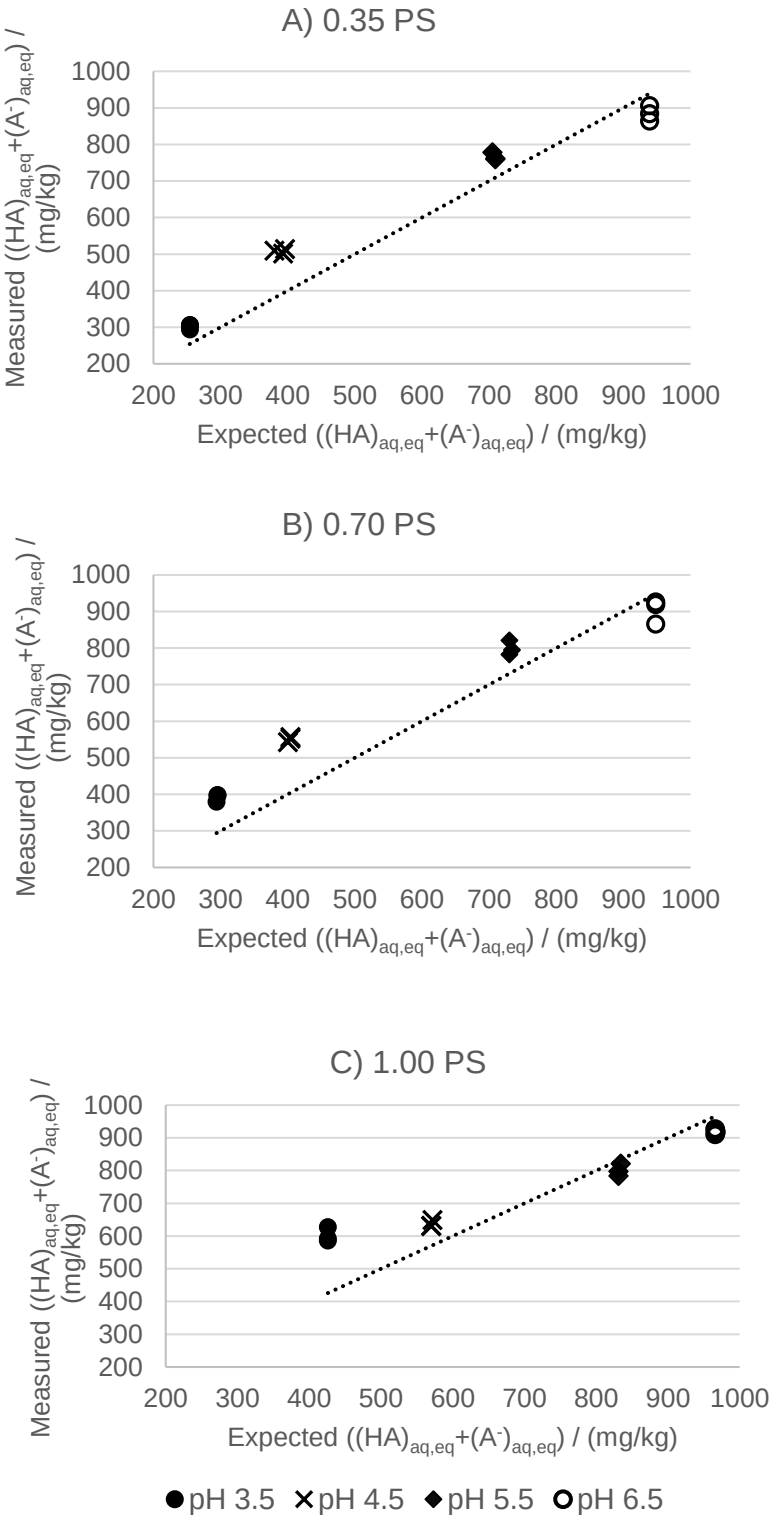


Figure 2.9 Expected versus measured ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) in after 8 weeks in model W|O+F systems of varying f_{tot}. Figures A,B,C show results in systems with 35, 70, 100% palm stearin, respectively. Model W|O+F systems were made in triplicate. HPLC injection performed in duplicate. The dotted trend line corresponds to an ideal situation, where measured values are equal to the expected values. Combined BF = 0.89. Combined AF = 1.17.

Figure 2.9 shows the $((HA)_{aq,eq}+(A^-)_{aq,eq})$ in model W|O+F systems after 8 weeks. It can be observed that the deviations of the expected from the measured data increased as f_{tot} increased. When combining the expected and measured results of the three data sets depicted in the aforementioned figure, the calculated BF was 0.89 and the AF was 1.17. This means that, when solid fat was present in the system, the model predicted lower values than were measured, by 11% in average. Also, for an expected sorbic acid concentration of 100 mg/kg in a model W|O+F system, the bounds expected to encompass approximately half of the observations would lie between 85-117 mg/kg.

Two explanations were assumed for this phenomenon. Considering that the trial period in these experiments was 8 weeks, and the model W|O+F systems were not shaken due to the solidification of the lipid phase, it can be assumed that equilibrium might have not yet been reached during the duration of the experiment, thus resulting in higher aqueous sorbic acid concentrations than expected. The second, and likely explanation, is that the K_p of the lipid phase changed when solid fat was added to the model emulsions. Namely, K_p values found in literature vary between oil types (see Chapter 1) and our calculations were performed with a fixed K_p of 3.3. In model W|O+F systems the fatty acid composition of the lipid phase no longer consists exclusively out of sunflower oil but of a mixture of sunflower oil and the liquid oil fraction of the palm stearine fat. We presumed that possibly the K_p of a solute will decrease as f_{tot} increases. To quantify this, we used the developed model to calculate the K_p which results in a BF of 0.98 and an AF of 1.08, as observed for model W|O systems at 22 °C (Figure 2.5 and 2.7). A K_p value of 2.45 was obtained, so the K_p value of the sorbic acid in W|O+F emulsions had to decrease from 3.3 to 2.45 to obtain the same prediction results as for W|O emulsions where no solid fat was present. A question of the influence of temperature on K_p was also prompted, but there seems to be little influence of varying temperature on K_p (Heinz, 1991).

It is important to note that the differences between measured and expected values were the largest in model W|O+F systems at pH 3.5, and the discrepancies increased as f_{tot} increased. Considering that at pH 3.5, sorbic acid is primarily present in its undissociated form which is the only form able to partition into the lipid phase, the partitioning process of the large amount of undissociated sorbic acid could have been retarded due to restraints imposed by the presence of solid fat. It is known that in foods such as butter and margarine, the dispersed aqueous phase is encased in a crystal network or by interfacially-adsorbed (“Pickering”) particles that promote emulsion stability (Rousseau et al., 2009). It was noticed, especially in the 0.35 PS model W|O+F system, that during prolonged storage solid fat crystals showed a tendency to accumulate on the interface of the system. This fat crystals sedimentation could

be the reason why diffusion of sorbic acid to the lipid phase was hindered. Expected versus measured results were also evaluated in the case that solid fat crystals allowed for the absorption of sorbic acid, i.e. it was assumed that solid fat wasn't inert. However, the discrepancies between the predicted and measured values were large in all cases, reaching up to 177,5% difference between the measured and expected values and systematically increasing with the increase of SFC in the system.

CONCLUSION

Sorbic acid remains an important mean to ensure the microbial stability and safety of W|O emulsions. In matrices of this type, growth occurs primarily in the aqueous phase and consequently the proportions of sorbic acid that migrates into the lipid phase is of no inhibitory importance.

Due to this complex situation, exact quantification of aqueous sorbic acid concentration is crucial, especially during product development.

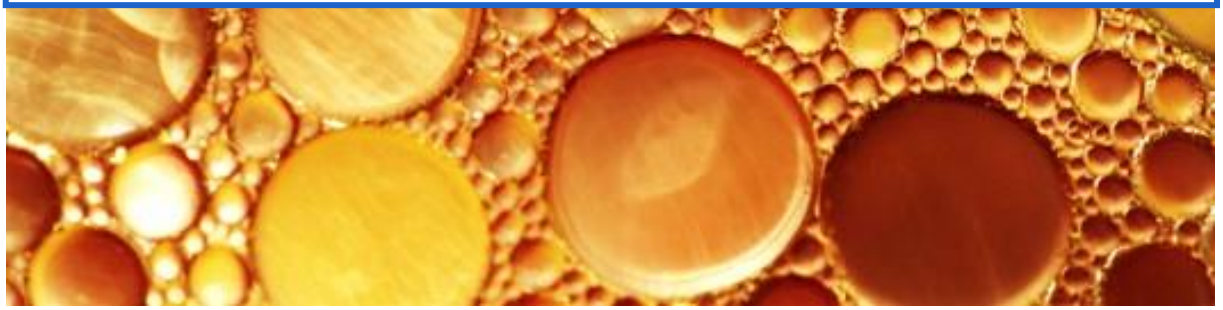
We have validated a model quantifying lipid and aqueous sorbic acid concentrations in W|O emulsions, taking into account pH, mass fraction of lipid phase and solid fat content. The measured data was in very good accordance with the expected values in model W|O systems. In model W|O+F systems, where a part of the lipid phase was crystalline, the deviations between the measured and expected data were slightly higher, but the $((HA)_{aq,eq}+(A^-)_{aq,eq})$ concentrations measured were generally higher than those expected. An important implication of the latter finding is the fact that the presence of solid fat will help retain the sorbic acid in the aqueous phase. In turn, this means that the concentration of active aqueous sorbic acid, $(HA)_{aq,eq}$, will be higher, but also that exposure time of sorbic acid on the microorganisms will be prolonged and thus the preservative will be more effective, in comparison with systems that contain only liquid oil. Practically, a higher solid fat content can be achieved either by lowering the temperature of the emulsion or by formulating the lipid phase with fats with a higher SFC content.

The proposed model can be used to quantify concentrations of any monoprotic weak organic preservative in model W|O systems.

To our best knowledge, the findings presented in this study are the first of their kind and are relevant for emulsion producing facilities.

CHAPTER 3

Effect of emulsification on sorbic acid distribution in model W|O+E systems



SUMMARY

The first step in the formation of a stabile emulsion is dispersion of one liquid phase in another liquid phase. A crucial factor in this emulsification process is the formation of a molecular layer at the W│O interphase by the emulsifier.

The aim of this study was to assess the influence of the emulsification process on the distribution of sorbic acid in model W|O+E systems, made as a simple representation of W│O emulsions. Sunflower oil and phosphate buffers of varying pH were used as the bulk phases of the model W|O+E systems. PGPR was used as the emulsifier and the model systems were prepared by emulsification with Ultra Turrax. Sampling was performed on four sampling days during one week and the model systems were centrifuged to obtain an isolated lipid phase. The sorbic acid concentrations in the centrifuged lipid phase were measured by HPLC and compared to the values obtained from a blank control (sorbic acid concentrations in lipid phase of a model W|O system subjected to the same emulsification procedure).

Slightly more sorbic acid was retrieved in the lipid phase of the centrifuged model W|O+E system than in the lipid phase of the model W|O system subjected to the same conditions. This could be explained as a consequence of the emulsification procedure itself and the larger surface area, maintained during time, between the lipid and aqueous phase in the model W|O+E system compared to the model W|O system. Nevertheless, due to the narrow experimental setup of the study it is difficult to draw a definite conclusion about the impact of emulsification on sorbic acid distribution in emulsions.

3.1. Introduction

The influence of pH, *r* and f_{tot} on the distribution of sorbic acid in model W|O+F systems was theoretically modelled and experimentally validated in Chapter 2. The aim of this experimental study was to investigate the effect of emulsification on sorbic acid distribution. For this purpose it was first necessary to (i) produce stabile model W|O+E systems and (ii) to obtain an isolated aqueous or lipid phase for sampling and comparison with model W|O systems. For the latter, a centrifugation step is used. The obtained results were then compared to theoretical values obtained from the model proposed in Chapter 2.

3.2. Materials and methods

3.2.1. Sorbic acid distribution in model W|O systems

A theoretical model describing the distribution of sorbic acid in model W|O systems was developed. Inhibitory aqueous sorbic acid concentrations and lipid phase sorbic acid concentrations, $(HA)_{aq,eq}$ and $(HA)_{lip,eq}$, were calculated over Equation 2.11 and 2.14 (See Chapter 2). For the development of the sorbic acid distribution model and further explanation about the variables in Equation 2.11, please refer to Chapter 2, Paragraph 2.2.1 and 2.2.2.

3.2.2. Preparation of aqueous phase in model W|O and W|O+E systems

The aqueous phases of model systems were prepared according to European Pharmacopoeia 7.0. (Council of Europe, 2004). Phosphate buffers were prepared and buffered at pH 3.5, 4.5, 5.5, 6.5, mimicking the pH range of oil and fat based products (Andersen and Williams, 1954; Roberts et al., 2005). Sorbic acid (1000 mg/L) was added to the phosphate buffers in the form of potassium sorbate. For the preparation of the phosphate buffers, refer to Chapter 2, Paragraph 2.2.2.1

3.2.3. Preparation of lipid phase in model W|O+E systems

Sunflower oil (Vandemoortele NV) was used as the lipid phase. 4000 mg/kg of PGPR on total system mass was added to sunflower oil and the mixture was placed on a magnetic stirrer with temperature control (IKA® RCT basic, IKA Werken, Germany) to mix at 200 rpm at 60 °C, 15 minutes before preparation of model W|O+E systems. The lipid phase of model W|O systems was comprised of only sunflower oil.

3.2.4. Preparation of model W|O and W|O+E systems

Model W|O+E systems (200 g) were prepared with 60% w/w phosphate buffer and 40% w/w sunflower oil with PGPR (4000 mg/kg on total system mass). Both phases were placed on a magnetic stirrer to heat until 60 °C. Emulsification of the systems was performed by slowly pouring the aqueous phase into the oil phase and mixing with Ultra Turrax (IKA T25, IKA Werken) for 5 minutes at 13500 rpm. The order of mixing the phases determines the character of the emulsion (i.e. adding water into oil produces a W│O emulsion). The character of the emulsion (W│O or O│W) was also verified via electrical conductivity (WTW cond 3310, Xylem Analytics Germany). The conductivity was below <100 µS/cm, characteristic for a W│O emulsion. The model W│O+E systems were then let to cool down at room temperature. Figure 3.1 shows the model W|O+E system produced as described above. The model systems were prepared in triplicate.

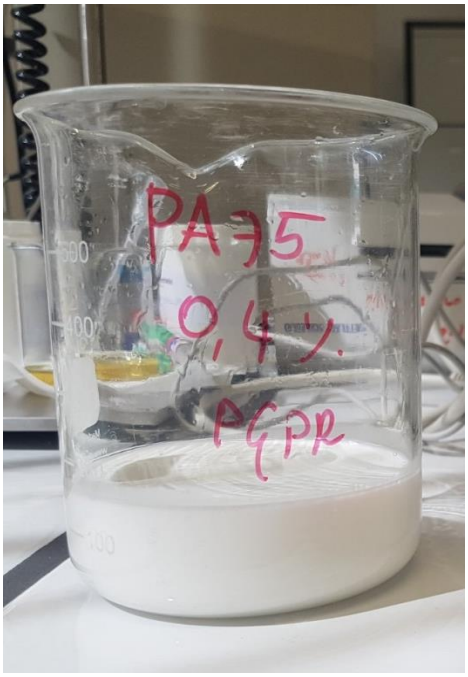


Figure 3.1. Model W|O+E system with 4000 mg/kg PGPR on total system mass. Emulsification performed with Ultra Turrax at 13500 rpm for 5 mins.

Model W|O systems without emulsifier were prepared in the manner described above. These systems were also prepared in triplicate and served as a blank control.

3.2.5. Centrifugation and sampling of model W|O+E systems

38 g of the model W|O+E system was placed into a plastic round bottom container and placed in a centrifuge (Avanti J26XP, Beckman Coulter, United States) for 30 minutes at 20000 rpm at 20 °C. The supernatant (5 g) was sampled with a Pasteur pipette and transferred to a 15 mL plastic capped test tube until extraction and HPLC analysis. Figure 3.2 shows the centrifuged model W|O+E system. The water content of the supernatant was also determined using ISO method 662:2016, by weighing 3-5 g of supernatant into an aluminum cup, leaving it for 16 hours at 103 °C and then weighing the sample. Six replicates were made and the water content of the supernatant was less than 1%.

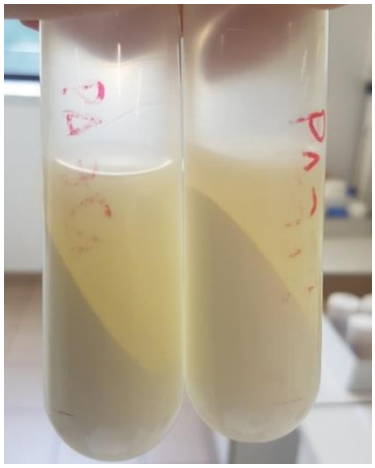


Figure 3.2. Model W|O+E systems centrifuged for 30 minutes at 20000 rpm and 20 °C.

3.2.6. HPLC analysis of sorbic acid in model W|O and W|O+E systems

Sampling was performed on days 0, 1, 5, 7. Around 0.5 g of supernatant of the model W|O+E systems as described in Paragraph 3.2.5. was weighed in a plastic test tube. Extraction solvent (5 mL) composed of 70% ethanol (Milipore, USA) plus 30% HPLC grade distilled water (VWR, USA) and 400 µL of 100 mg/L vanillin solution (Merck, USA) was added. The 100 mg/L vanillin solution was used as internal standard. This mixture was was filtered through a 0.2 µm DynaGard (DYNA Instruments, Germany) filter into an HPLC vial (Agilent Technologies, USA). Chromatographic conditions of the analysis are described in Chapter 2, Paragraph 2.2.2.4. In the case of the W|O systems without emulsifier (i.e. blank control), both the aqueous and lipid phase were sampled. From the aqueous phase of the W|O food model system, 50 µL was sampled and brought in an HPLC vial with 450 µL of the extraction solvent and 500 µL of 100

mg/L vanillin solution. The extraction and chromatographic analysis of sorbic acid from the lipid phase of the W|O system was performed as described above.

3.2.7. Statistical analysis

Minitab® 17.1.0 was used for basic data exploration. Normality was tested with the Anderson-Darling normality test. Homoscedasticity was tested with Bartlett's test. In the case of equality of variances, a one-way ANOVA was used to assess differences in sorbic acid concentrations during time. In case of non-equal variances, Welch's t-test and Games-Howell Pairwise Comparisons were used to assess the differences between the measured mean sorbic acid concentrations. The differences between $(HA)_{lip,eq}$ measured in the model W|O+E systems and $(HA)_{lip,eq}$ measured in the model W|O systems were assessed by 2-sample t-tests. A 5% significance level was applied for all statistical tests.

3.3. Results and discussion

3.3.1. $((HA)_{aq,eq}+(A^-)_{aq,eq})$ and $(HA)_{lip,eq}$ in non-emulsified model W|O systems

The initial concentration of sorbic acid in the aqueous phase, $(HA)_{aq,i}$, was 1000 mg/L (1340 mg/L potassium sorbate). Considering that both the model W|O systems and W|O+E systems were made with 40% w/w sunflower oil and 60% w/w phosphate buffer, this means that the total concentration of sorbic acid in the systems, $(HA)_{tot,eq}$, was ca. 600 mg/kg. When comparing the two graphs in Figure 3.3, it can be seen that at pH 3.5, where 95% of sorbic acid is undissociated ($pK_{a(sorbic\ acid)}$ = 4.75), most of the acid partitioned into the lipid phase.

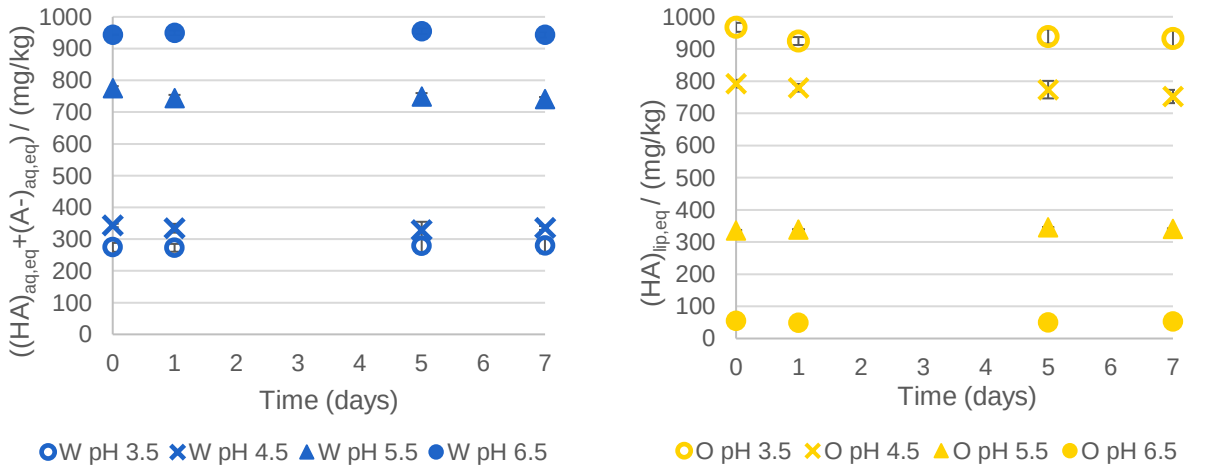


Figure 3.3. Measured sorbic acid $((HA)_{aq,eq}+(A-)_{aq,eq})$ (left) and $(HA)_{lip,eq}$ (right) concentrations during time in model W|O systems of varying pH. Error bars denote standard deviation.

It can be seen from Figure 3.3. that, the higher the pH, the more sorbic acid was retrieved in the water phase. However, with the increase of pH, there is an increase in the amount of the dissociated form of sorbic acid, i.e. at pH 4.5, 5.5, 6.5, there is 36, 85, 98% of dissociated sorbic acid present in the system, respectively. Effectively, this means that at a relatively high pH, like 6.5, almost all the acid will remain in the water phase, but it will exert little inhibitory activity. It can also be seen that at pH 4.5, the measured $((HA)_{aq,eq}+(A^-)_{aq,eq})$, were lower in comparison with $((HA)_{aq,eq}+(A^-)_{aq,eq})$ in model W|O systems of other pH values. The differences between $((HA)_{aq,eq}+(A^-)_{aq,eq})$ at sampling days 0,1,5,7 and $(HA)_{lip,eq}$ at sampling days 0,1,5,7 in model W|O systems at each pH were assessed. There were no significant differences ($p>0.05$) between the means of measured $((HA)_{aq,eq}+(A^-)_{aq,eq})$ during time and $(HA)_{lip,eq}$ during time. Effectively, this means that the partitioning of sorbic acid occurred immediately after mixing the aqueous and lipid phase with Ultra Turrax for 5 mins at 13500 rpm and the measured concentrations didn't change during 7 days.

In Chapter 2, a model describing the distribution of sorbic acid in model W|O systems was developed in Microsoft® Excel®. Figures 3.4 and 3.5 show the measured versus expected sorbic acid concentrations in the lipid and aqueous phase of model W|O systems, $(HA)_{lip,eq}$ and $((HA)_{aq,eq}+(A^-)_{aq,eq})$, respectively. Final pH values of the water phases of all experimental conditions are listed in Table 3.1.

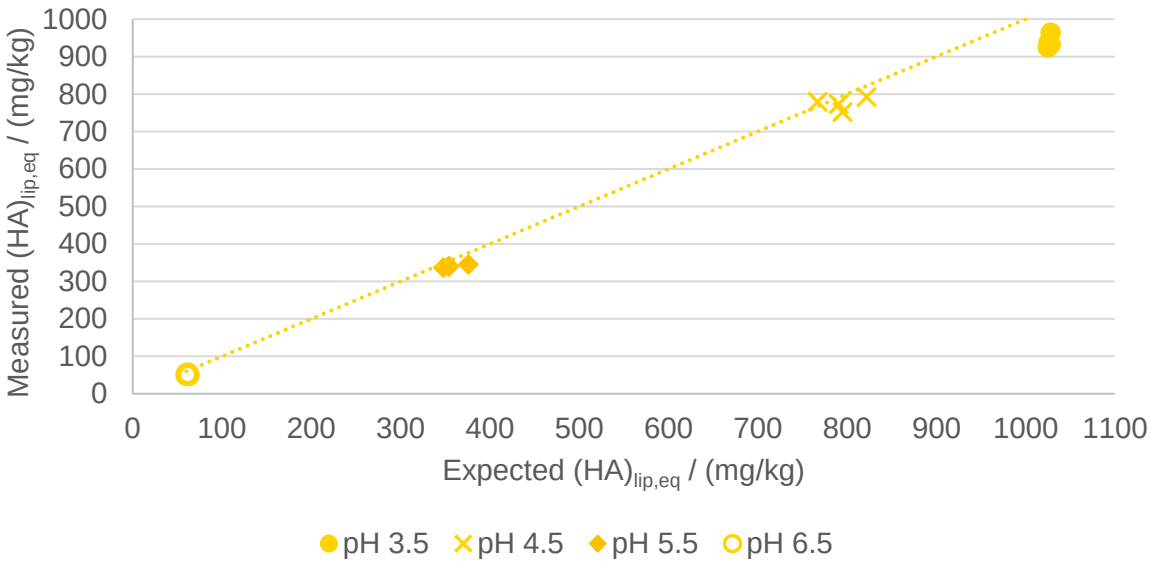


Figure 3.4. Measured versus expected sorbic acid concentrations in oil phase of model W|O systems, $(HA)_{lip,eq}$. Data points are averages of triplicates on each sampling day. HPLC injection performed in duplicate. The dotted trend line corresponds to an ideal situation, where measured values are equal to the expected values. BF=0.92, AF=1.09.

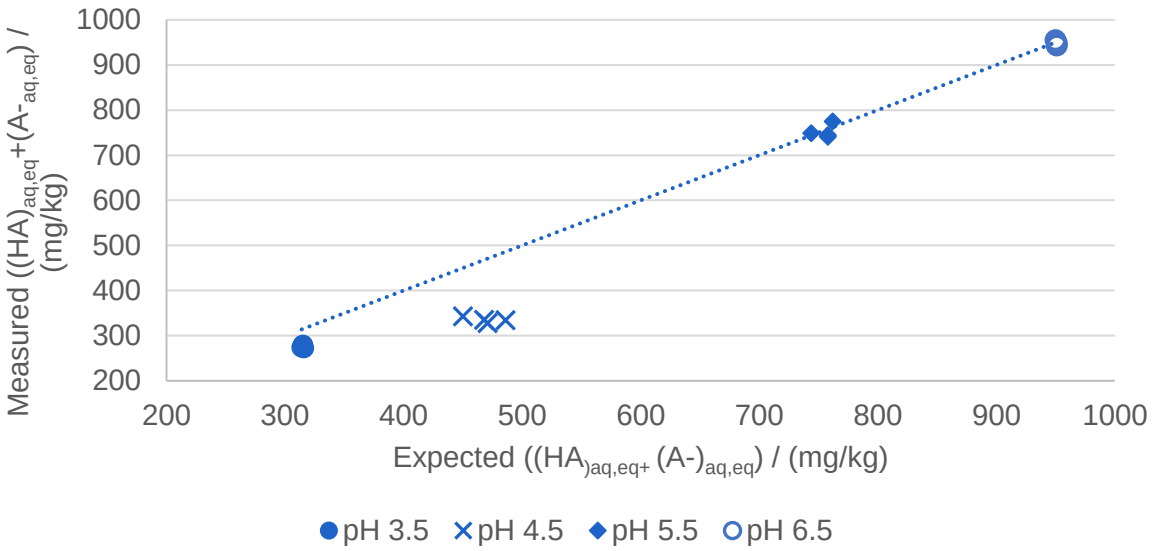


Figure 3.5. Measured versus expected sorbic acid concentrations in water phase of model W|O systems, $((HA]_{aq,eq}+(A-)_{aq,eq})$. Data points are averages of triplicates on each sampling day. HPLC injection performed in duplicate. The dotted trend line corresponds to an ideal situation, where measured values are equal to the expected values. BF=0.89, AF=1.13.

Table 3.1. Measured pH values in aqueous phases of model W|O systems at four sampling days. Average and standard deviation are calculated from 3 replicates.

Measured pH	pH 3.5	pH 4.5	pH 5.5	pH 6.5
Day 0	3.75 ± 0.03	4.75 ± 0.02	5.60 ± 0.01	6.50 ± 0.01
Day 1	3.80 ± 0.02	4.87 ± 0.02	5.59 ± 0.02	6.51 ± 0.04
Day 5	3.78 ± 0.04	4.82 ± 0.01	5.55 ± 0.03	6.49 ± 0.01
Day 7	3.76 ± 0.01	4.81 ± 0.03	5.59 ± 0.03	6.51 ± 0.02

The measured concentrations in Figure 3.4 show a very close correspondence to the proposed model. The recovery of sorbic acid by HPLC was from 0.83 (pH 6.5) to 1.04 (pH 3.5). The relatively low recovery at pH 6.5 could have been a consequence of the low $(HA)_{lip,eq}$ concentrations that were more prone to measurement error. The AF and BF calculated for Figure 3.4 are closer to 1 than the AF and BF calculated for Figure 3.5. We presumed this is due to the exact knowledge of sample masses in the lipid phase while calculating the final measured concentration (i.e. lipid samples were weighed on an analytical scale with ± 0.0001 g precision). The samples from the aqueous phase, on the other hand, were sampled with a pro-pipette (20-100 µL capacity) and mixed in a primarily ethanol based (volatile) extraction solvent. Although the recommended pipetting technique for volatile compounds was followed (i.e. pre-rinsing the pipette tip to saturate it with ethanol vapor, followed by reverse pipetting), the exact volumes of the aqueous phase and extraction solvent could have slightly varied. Nevertheless, as elaborated in Chapter 2, it can be concluded that sorbic acid concentrations in a model W|O system are dependent on the pH of the system.

3.3.2. $(HA)_{lip,eq}$ in model W|O+E systems

Strong surfactants are needed to stabilize microstructured fluids like emulsions. PGPR is a non-ionic, oil-soluble surfactant that is described as one of the most potent surfactants in stabilizing W|O emulsions (Wolf and Koehler, 2012; Norton et al., 2013). In this study, a level of 4000 mg/kg of PGPR on total system mass was applied, as specified in EC EU 1333/2008 as the maximum amount allowed. In order to determine if emulsification of a W|O emulsion influences sorbic acid partitioning, it was needed to centrifuge the model W|O+E system to obtain at least one individual phase. The sorbic acid concentrations measured in this isolated phase would be compared to concentrations measured in the corresponding individual phase of the model W|O system, subjected to the same emulsification procedure. The phase that separated was the lighter (i.e. lower density than water) lipid phase and it was not possible to isolate the aqueous phase which remained trapped in the white creamy sediment on the bottom of the centrifugation tube (Figure 3.2). Figure 3.6 shows that $(HA)_{lip,eq}$ from the centrifuged model W|O+E systems were slightly higher than the $(HA)_{lip,eq}$ from the lipid phase of the model W|O systems. More specifically, 8, 14, 15, 29% more of $(HA)_{lip,eq}$ was retrieved in model W|O+E systems than in model W|O systems at pH 3.5, 4.5, 5.5, 6.5, respectively, at day 7.

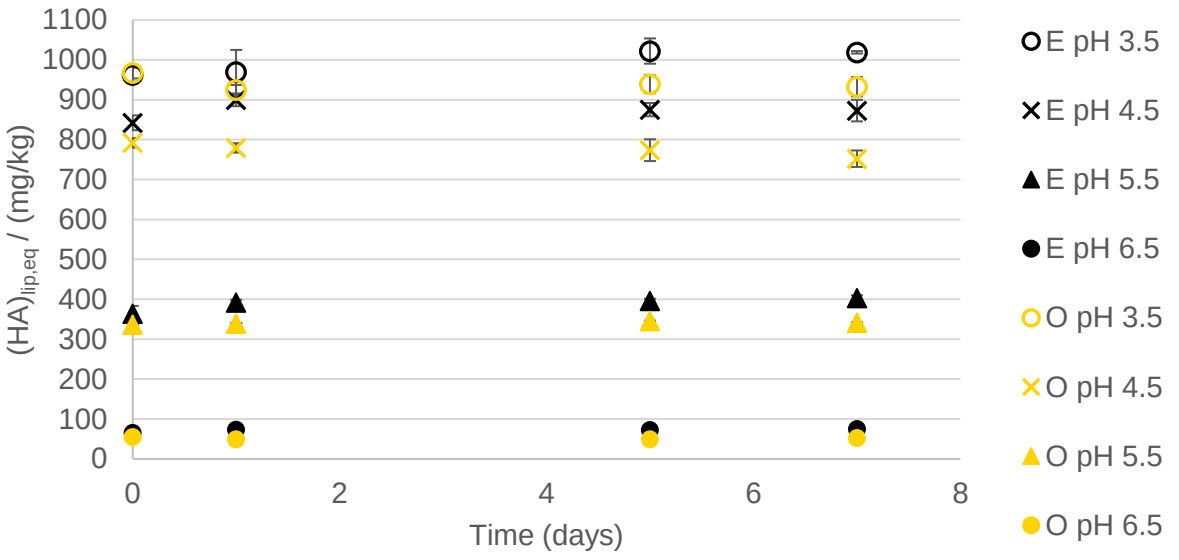


Figure 3.6. Measured sorbic acid concentrations during time, $(HA)_{lip,eq}$, in model W|O systems (yellow data points) and model W|O+E systems (black data points) of varying pH. Error bars denote standard deviation.

The measured $(HA)_{lip,eq}$ were assessed for statistically significant differences with 2 sample t-tests where non-equal variances were assumed. Comparisons were made per pH and per day (i.e. $(HA)_{lip,eq}$ from W|O emulsion at pH 3.5 day 0 vs. $(HA)_{lip,eq}$ from W|O+E emulsion at pH 3.5 day 0).

The results implied that emulsification of a model W|O+E system could have influenced the partitioning of sorbic acid, where slightly more sorbic acid was present in the lipid phase of the above mentioned system when compared to a model W|O system. Nevertheless, due to the small differences in the extracted $(HA)_{lip,eq}$ concentrations between the model W|O+E and W|O systems, and the narrow experimental design of the study, it is difficult to draw a definite conclusion. The first step in the formation of a stabile emulsion is dispersion of one liquid phase in another liquid phase. A crucial factor in this emulsification process is the formation of a monomolecular layer at the lipid/aqueous interphase by the emulsifier. During emulsion formation there is a large increase in surface area (up to several thousand fold), dependent upon the number and size of the droplets (Branen et al., 2001). The fact that the surface area between the aqueous and lipid phase was substantially increased in model W|O+E systems when compared to model W|O systems, could have contributed to the increased concentration of sorbic acid in the lipid phase of the model W|O+E systems.

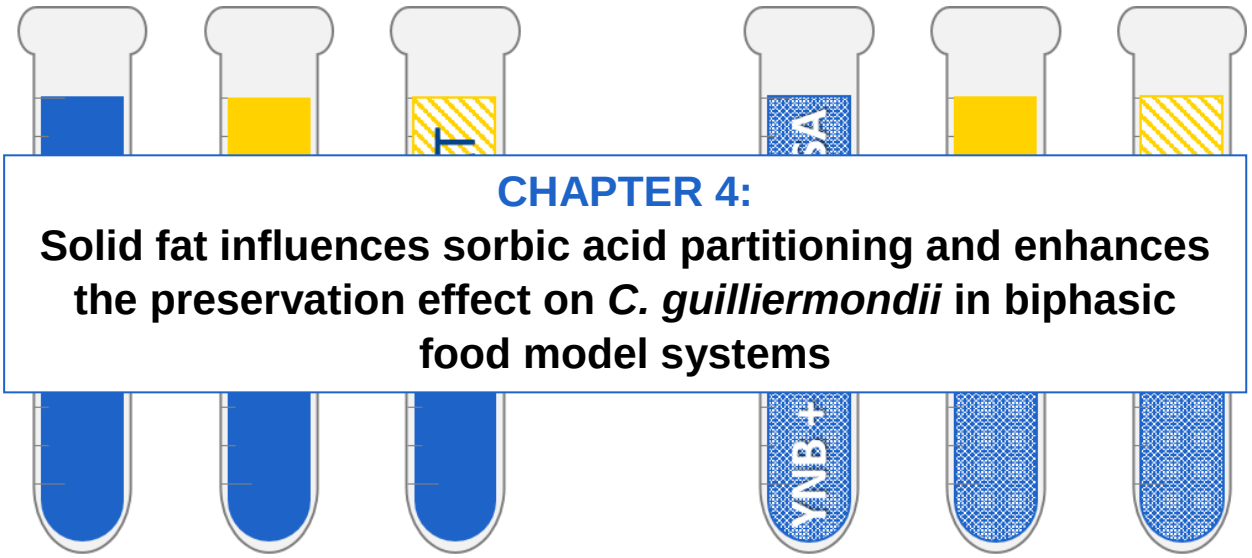
At pH 3.5, significant differences were observed at day 5 and 7, but not at days 0 and 1. At this pH, there is a large amount of protonated sorbic acid present that is able to partition into the lipid phase, when compared to the higher pH values used in this experimental study. Riviere (2017) stated that the rate of diffusion of a compound across a (lipid-based) membrane is directly proportional to its concentration gradient across the membrane, lipid/water partition coefficient and diffusion coefficient. Pérez-Isidoro et al. (2014) claimed that high protonation rates of anesthetics reduce their diffusion speed into lipid membranes. The model W|O+E systems could be perceived as a simple representation of a biological lipid membrane. Considering the elevated undissociated sorbic acid concentration involved in the process of partitioning at pH 3.5, it is possible that this relatively high undissociated sorbic acid concentration influenced the rate of diffusion of the compound by retarding the process. From the increase in $(HA)_{lip,eq}$ differences mentioned above, one might conclude that these differences increase proportionally to pH. However, we suspect this is not the case and the increase in differences of measured sorbic acid concentrations in model W|O and W|O+E systems between sampling days at higher pH's could have been attributed to the generally lower concentration values of $(HA)_{lip,eq}$, where these lower values could have been more prone to variability caused by experimental error.

CONCLUSION

Emulsion science is a multi-disciplinary subject covering chemistry, physics and engineering. Emulsifiers are added to emulsions to increase product stability and attain an acceptable shelf-life. The function of an emulsifier is to join together the immiscible phases of an emulsion in a homogenous and stabile preparation. PGPR is a non-ionic surfactant used in the preparation of W│O emulsions.

The aim of this experimental study was to investigate the effect of emulsification on the distribution of sorbic acid. For this to be investigated, a method to produce stabile model W|O+E systems which could be separated by centrifugation was first developed.

The results showed that emulsification could have an effect on the distribution of sorbic acid. Slightly more $(HA)_{lip,eq}$ was present in a model W|O+E system when compared to a model W|O systems. This phenomenon could be explained by the increase of the surface area of the W│O interphase when the emulsification procedure is applied, which consequently gives a larger surface for the sorbic acid partitioning phenomenon to occur. Nevertheless, due to the narrow experimental setup of the study it is difficult to draw a definite conclusion about the impact of emulsification on sorbic acid distribution in emulsions.



CHAPTER 4:
Solid fat influences sorbic acid partitioning and enhances the preservation effect on *C. guilliermondii* in biphasic food model systems

This chapter is published as:

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SUMMARY

The influence of liquid oil and solid fat on sorbic acid distribution and preservation effect was investigated in food model systems.

The aqueous phase of the food model systems was comprised of Yeast Nitrogen Broth (YNB) in phosphate buffer and buffered at pH 3.5, 4.5, 5.5, 6.5. Sorbic acid (100 mg/L) in the form of potassium sorbate was added to the aqueous phase. *Candida guilliermondii* (2 log CFU/mL) was inoculated in the water phase to resemble industrial post-contamination CFU levels. Growth parameters, generation time (GT) and lag phase (λ) of *C. guilliermondii* in the water phase of the food model systems were quantified during 1 month at 7°C and maximum 80 hours at 22°C.

HPLC analyses were performed to evaluate $((HA)_{aq,eq}+(A^-)_{aq,eq})$ in each food model system. Sorbic acid inhibited growth of *C. guilliermondii* in YNB at pH 3.5 and 4.5 at 7 °C and 22 °C. The presence of liquid oil in YNB+SA│oil systems caused partitioning of sorbic acid into the lipid phase at pH 3.5 and 4.5 at 7 °C and 22 °C, reducing its inhibitory effect. Adding solid fat into the model YNB+SA│oil+fat system significantly prolonged λ at pH 4.5 at 22 °C and growth was inhibited at 7 °C. HPLC analysis showed 34 mg/kg and 44 mg/kg $((HA)_{aq,eq}+(A^-)_{aq,eq})$ in YNB+SA│oil at pH 4.5 at 7 °C and 22 °C after 1 month and 168 hours, respectively. In YNB+SA│oil+fat at pH 4.5, 87 mg/kg $((HA)_{aq,eq}+(A^-)_{aq,eq})$ was measured after 1 month at 7 °C and 168 hours at 22 °C, indicating that the presence of solid fat retards sorbic acid partitioning.

Results showed that liquid oil and solid fat have an effect on $((HA)_{aq,eq}+(A^-)_{aq,eq})$ and its preservation effect. The presence of solid fat reduced the tendency of sorbic acid to partition into the lipid phase, which was reflected in the increased inhibitory effect of sorbic acid on *C. guilliermondii.*

4.1. Introduction

The distribution behaviour of sorbic acid in different systems has been investigated by several researchers (Deuel et al., 1954a,b; Lubieniecki-von Schelhorn, 1967; Sofos, 1989). Despite the several studies that have been performed to date, none have comprehensively investigated the influence of type of lipid (oil and/or fat) in an emulsion on the partitioning and effectiveness of sorbic acid. The experimental investigation of partitioning of sorbic acid in aqueous, oil and fat systems is fundamentally valuable for optimising its applications in emulsions.

In this work, the influence of sunflower oil (liquid oil) and a palm stearin hardstock (solid fat) on the distribution behaviour of sorbic acid and its preservation effect in model emulsions was experimentally determined by means of HPLC analysis and validated by challenge tests with *C. guilliermondii*. The aqueous phase consisted of phosphate buffer and nutritive media, buffered at pH 3.5, 4.5, 5.5, 6.5, to simulate the typical pH values of emulsions. *C. guilliermondii* was chosen as a model spoilage yeast. Preliminary tests (results not shown) showed that *C. guilliermondii* was completely inhibited by 300 mg/L sorbic acid. As the aim of the study was to demonstrate the effect of sorbic acid on the λ and GT of *C. guilliermondii*, 100 mg/L of sorbic acid was chosen as the concentration that would permit growth and enable quantification of the growth parameters.

4.2. Materials and methods

4.2.1. Yeast strain and culturing conditions

C. guillermondii, strain NP566U + pURA5-GFP, was kindly provided by Dr. N. Papon of the Biomolécules et Biotechnologies Végétales, Université François-Rabelais de Tours, Faculté de Pharmacie, Tours, France. Stock cultures were prepared as follows. Firstly, sub-cultures were prepared by incubating 5 mL Yeast Nitrogen Broth (YNB) inoculated with *C. guilliermondii* for 48 hours at 22 °C. Thereafter, these sub-cultures were streak plated on Yeast Nitrogen Agar (YNA) and incubated at 30 °C for 5 days to obtain the stock cultures which were subsequently stored at 4 °C until required. Stock cultures were prepared monthly. YNB consisted of 6.7 g Yeast Nitrogen Base without amino acids (Sigma Aldrich, USA) and 20 g of saccharose (Sigma Aldrich) in 1 L of distilled water. YNA had the same composition as YNB, with 20 g of bacteriological agar (Sigma Aldrich) added to solidify the medium. YNB was filter sterilized through a Rapid Flow Bottle Top Filter (Nalgen, USA) with a pore diameter of 0.45 µm. YNA was sterilized by autoclaving for 15 minutes at 121 °C.

4.2.2. Preparation of inoculum for challenge tests

C. guilliermondii was grown in 5 mL YNB at 22 °C for 48 hours while shaking at 200 rpm on a plate shaker (IKA Werken, Germany). Shaking (agitation) had previously been determined to increase the cell yield by *ca.* 1 log CFU/mL in comparison to non-shaken (static) cultures. Cell counts of 8 log CFU/mL were obtained in these stock suspensions after 48 hours of incubation. The inoculum for challenge tests was prepared by dilution of the stock suspension in buffered YNB to *ca*. 3 log CFU/mL.

4.2.3. Preparation of aqueous phase in model W | O emulsions

The aqueous phase of model W|O emulsions consisted of phosphate buffer supplemented with nutrients. Phosphate buffers at pH 3.5, 4.5, 5.5, 6.5 were prepared according to European Pharmacopoeia (Council of Europe, 2004). All materials used to make phosphate buffers were obtained from Chem-Lab NV, Belgium. Yeast Nitrogen Base without amino acids (6,7 g/L) as well as saccharose (20 g/L) were added to the buffers. Potassium sorbate (VWR, USA), 134 mg/L, corresponding to 100 mg/L (100 ppm) of sorbic acid (SA), was added to each buffer in the experiments investigating the effect of sorbic acid on the growth of *C. guilliermondii*. The pH of these buffers was checked and adjusted after the addition of the nutrients and potassium sorbate with H_3PO_4 (6M) and NaOH (10M) (Merck, USA). The pH of the buffers was measured and adjusted at 20 °C with the aid of a digital pH meter (S220 SevenEasy, Mettler-Toledo, USA).

4.2.4. Preparation of model W|O systems

A ratio of 40% aqueous phase and 60% lipid phase by weight was used in all conditions. None of the samples were emulsified, as it was assumed that the addition of the emulsifier would influence sorbic acid behavior (see Chapter 3). The aqueous phases were combined with the diluted stock suspension of *C. guilliermondii* to achieve an initial concentration of *ca.* 2 log CFU/mL. Thereafter, 15 g of sunflower oil (Vandemoortele NV, Belgium) was added to 10 mL of each aqueous phase. Model W|O systems were prepared by combining 7.5 g of sunflower oil and 7.5 g of a solid interesterified hard stock fat (Vandemoortele NV) which were heated up to 60 °C and mixed at 10000 rpm for 3 minutes by means of an Ultra Turrax (IKA T25 Digital, Germany). The oil+fat phase mixture was then allowed to cool to 37 °C before placing it onto the inoculated aqueous phase in order to prevent potential thermal inactivation of the inoculum. All model W|O emulsions were prepared in triplicate and incubated at 7 °C and 22 °C for 1

month and maximum 80 hours, respectively. A schematic representation of the model systems can be seen in Figure 4.1.

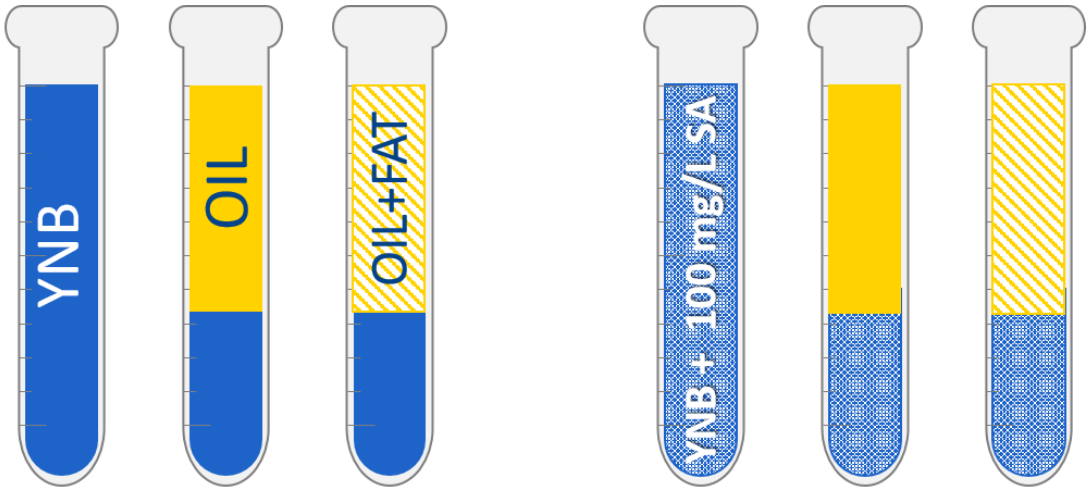


Figure 4.1. Model W|O emulsions. Tubes with oil and/or fat are comprised of 40%, (i.e.10 mL) water phase and 60% (i.e.15 g) oil and/or fat. In model systems with fat, the oil:fat ratio is 50:50. *C. guilliermondii* was inoculated in all tubes at approximately 2 log CFU/mL.

4.2.5. Sampling and enumeration of *C. guilliermondii*

Sampling was performed periodically during storage by aseptically drawing 100 µL aliquots from the aqueous phase of each replicate. This small sample volume was drawn from the replicates to ensure a suitably large volume of aqueous phase remained in the Falcon flasks to permit further sampling. It should be noted that an equivalent volume of oil was also removed to maintain the 40:60 ratio of aqueous to lipid phase. Serial decimal dilutions were prepared in Peptone Physiological Salt (PPS, 8.5 g NaCl + 1 g neutralized bacteriological peptone per L). In the case of YNB(+SA)|oil+fat systems, repeated sampling from the same recipient was not possible as the aqueous phase could only be sampled after the lipid phase had been destroyed. Therefore, in those cases, 12 replicates in triplicate were prepared for each pH. Sampling was performed in regular intervals during incubation for maximum 80 hours in the case of the samples placed at 22 °C and during 1 month in the case of samples placed at 7 °C. Serial dilution of the samples was performed by means of a spiral plater (Eddy Jet, USA) after which the appropriate dilutions were plated out on YNA. The plates were incubated at 30 °C for 5 days and colonies were counted with the aid of the Eddy Jet counting grid. In the case of enumerating a high dilution, all the colonies on the plate were counted. All data are expressed as log CFU/mL in the water phase of the system. Equation 4.1 shows the calculation of CFU/mL from Eddy Jet plates.

$$\frac{CFU}{mL} := \frac{\sum colonies\ in\ area}{\bigvee grid\ area/mL} \quad (4.1)$$

4.2.6. Determination of generation time (GT) and lag phase duration (λ)

To estimate the generation times and lag phase durations at each experimental conditions, the growth function of Baranyi and Roberts (1994), Equation 4.2, was fitted to the experimental data (log CFU/ml as a function of time) by means of the DMFit freeware tool embedded in the ComBase Modelling Toolbox.

$$y(t) = y_0 + \mu_{max} A(t) - \frac{1}{m} ln\left(1 + \frac{e^{m\mu_{max}A(t)} - 1}{e^{m(y_{max}-y_0)}}\right) \quad (4.2)$$

Where μ_{max} (1/time) is the maximum specific growth rate; y_0 = ln $x(t_0)$, the natural logarithm of the cell concentration at $t=t_0$; y_{max}= ln x_{max}, the natural logarithm of the maximum cell concentration and m is a curvature parameter characterizing the transition of the growth curve to the stationary phase.

The function A(t) (Equation 4.3) plays the role of a gradual delay in time:

$$A(t) = t + \frac{\ln(e^{m\mu_{max}t} + e^{-h_0} - e^{-\mu_{max}t-h_0})}{\mu_{max}} \quad (4.3)$$

where h_0 = -ln α_0 and α_0 is the physiological state of the cells at $t=t_0$.

μ_{max} was then recalculated to generation time according to Equation 4.4.

$$GT = \frac{\log(2)}{\mu_{max}} \quad (4.4)$$

where GT is generagtion time (1/h) (Cooper, 1991).

4.2.7. HPLC analysis of sorbic acid in model W | O systems

Samples of model W | O systems were prepared in as described in Paragraph 4.2.4. As far as YNB+SA | oil+fat systems are concerned, only those at pH 4.5, 5.5 and 6.5 were sampled, because growth was inhibited in YNB at pH 3.5. Firstly, inoculated samples were prepared in

triplicates and only sampled at the beginning and end times of the microbiological trials, i.e. maximum 80 hours for samples kept at 22 °C and 1 month for samples kept at 7 °C, to determine if *C. guilliermondii* consumes sorbic acid. It was determined that it did not consume sorbic acid. Thereafter, non-inoculated samples were prepared in triplicates and incubated at 22 °C and 7 °C. The samples stored at 22°C were sampled at 0, 24, 48, 72, 96 and 168 h of incubation. The incubation period in this part of the study was longer than that used in the growth study to observe the time dependent partitioning of sorbic acid. Samples kept at 7 °C were sampled at days 0, 1, 3, 7, 14, 23 and 30. To determine the sorbic acid concentration in the water phase of the samples, 500 µL of water phase was transferred to an HPLC vial (Agilent Technologies, USA) and mixed with an equal volume of extraction solvent consisting of 70% of ethanol (Milipore, USA) and 30% HPLC grade distilled water (VWR, USA). HPLC analysis of the samples was performed as described by Pylypiw and Grether (2000). For chromatographic conditions, refer to Chapter 2, Paragraph 2.2.2.4.

4.2.8. Solid fat content (SFC) measurements

SFC was measured from the stock oil+fat mixture using a Maran Ultra 23 MHz pulsed Nuclear Magnetic Resonance (pNMR) Analyzer (Oxford Instruments, UK) after the microbiological evaluations. These mixtures were kept in closed 2 L glass jars on room temperature and dark place. The NMR-tubes were filled with the oil+fat mixtures and kept at 7 °C or 22 °C several days before analysis. These tubes were then placed in a water bath at 5 °C for 1 hour before measurement. Using calibration standards (0.0, 29.3, 70.5% SFC), SFC values were derived. SFC measurements of the oil+fat lipid phases revealed 19.7 and 33.0% of SFC at 22 °C and 7 °C, respectively.

4.2.9. Statistical analysis

Minitab® 17.1.0 was used for basic data exploration. GT's and λ among the different factors involved in this study were compared and a 5% significance level was applied for all statistical tests. Normality was tested over normal probability plots. Homoscedasticity was tested with F-tests. Considering that the explanatory variables were categorical (pH and oil or fat content) and the response was quantitative (CFU/mL or $((HA)_{aq,eq}+(A\text{-})_{aq,eq})$ / (mg/kg)), an ANOVA with a Tukey's post-hoc test were performed to compare sample means and establish where significant differences occurred.

4.3. Results and discussion

4.3.1. Influence of pH on sorbic acid behavior and growth of *C. guilliermondii*

The growth curves obtained depicting the effect of sorbic acid and the presence of oil and/or fat at pH's 3.5-6.5 on the growth of *C. guilliermondii* are shown in Figures 4.2-4.5. The estimated generation times (GT, 1/h or d) and lag phases (λ, h or d) are shown in Table 4.1. As can be observed from Figures 4.2 and 4.3, in YNB systems at 22 °C, both the GT and the λ were significantly different at pH 3.5 when compared to pH 4.5 ($p < 0.05$). The calculated GT was 5.54 ± 0.70 1/h compared to 3.63 ± 0.07 1/h and the lag phase duration was 10.23 ± 3.23 hours compared to 2.84 ± 0.89 hours at pH 3.5 and pH 4.5, respectively.

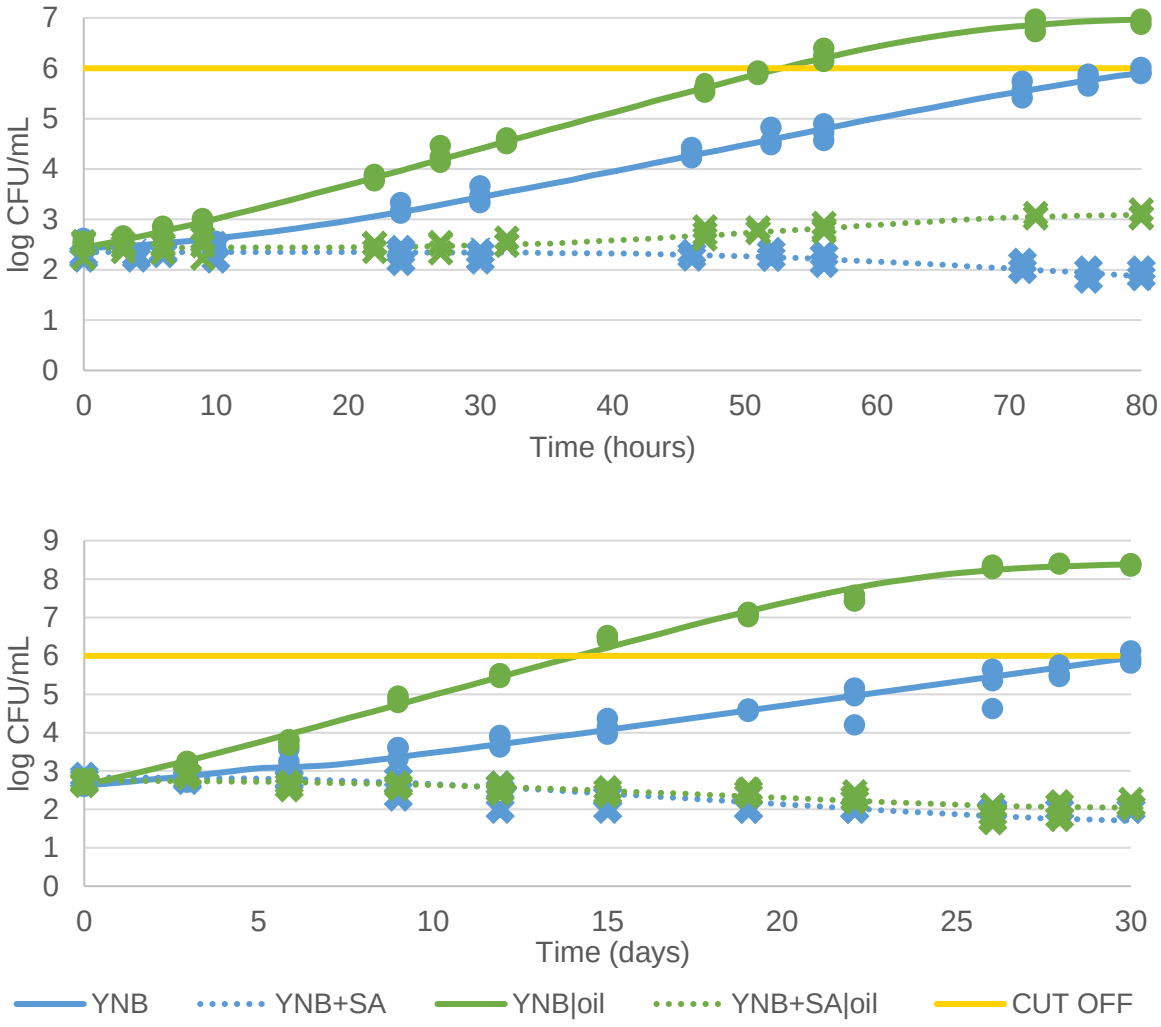


Figure 4.2. Dashed and full lines represent fitted growth curves of *C. guilliermondii* in YNB(+SA) and YNB(+SA)|oil systems at pH 3.5 at 22 °C (top) and 7 °C (bottom). × and ● symbols represent sampling points from three replicates of systems with and without 100 mg/kg sorbic acid (SA) (i.e. 134 mg/kg potassium sorbate), respectively, as obtained by plate counts. A cut-off level at 6

log CFU/g is chosen as the point of spoilage and development of off-flavours. R^2>90% for all fittings.

At 7 °C, the GT of YNB systems at pH 3.5 was significantly prolonged compared to YNB systems at pH 4.5, although the calculated lag phase duration wasn't different. This might be due to the large standard deviation associated with the mean lag phase duration of YNB systems at pH 3.5. These results indicate that the growth of *C. guilliermondii* in an unpreserved system could have been retarded by low pH.

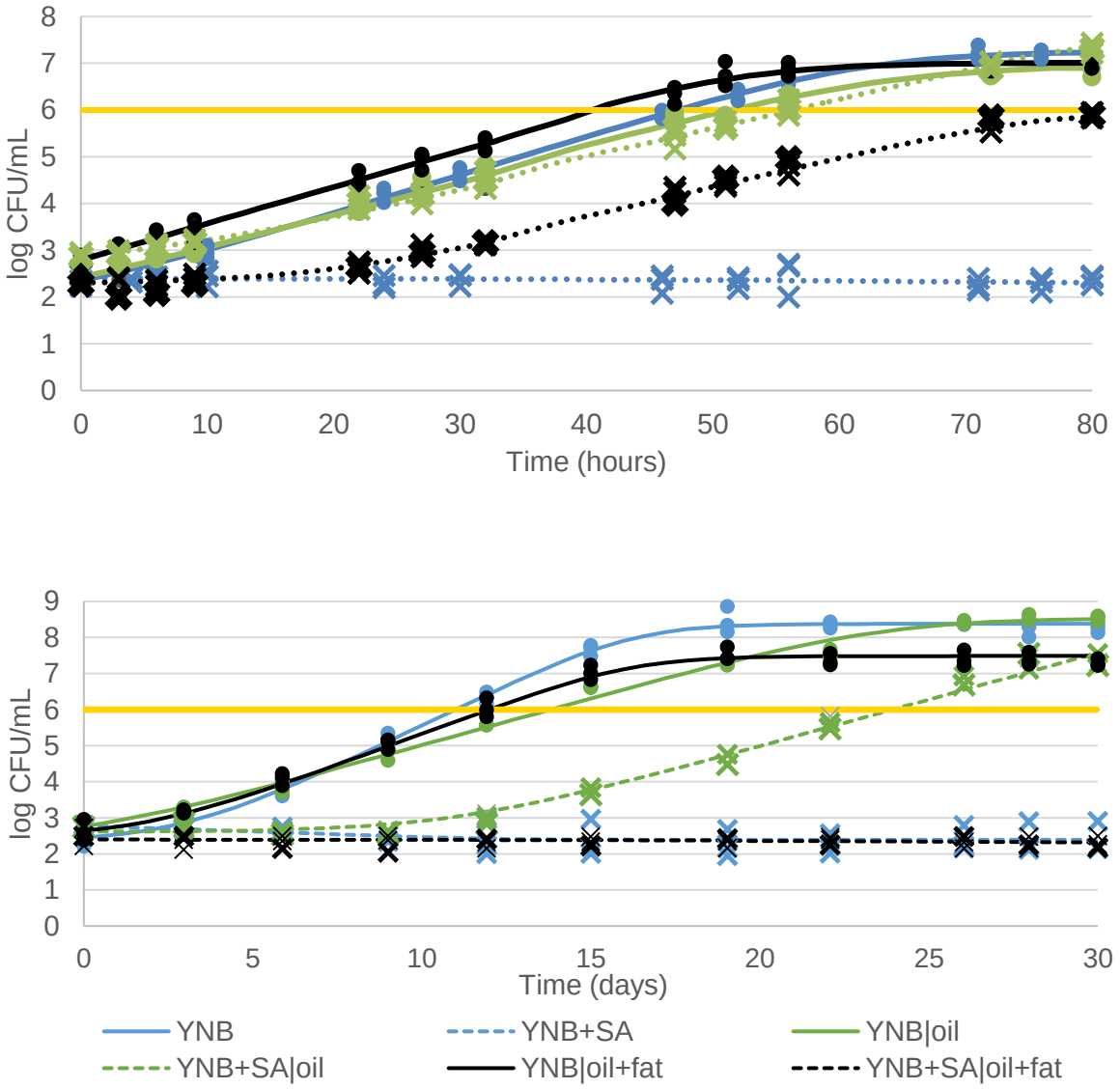


Figure 4.3. Dashed and full lines represent fitted growth curves of *C. guilliermondii* in YNB(+SA), YNB(+SA)|oil and YNB(+SA)|oil+fat systems at pH 4.5 at 22 °C (top) and 7 °C (bottom). × and • symbols represent sampling points from three replicates of systems with and without 100 mg/kg sorbic acid (SA) (i.e. 134 mg/kg potassium sorbate), respectively, as obtained by plate counts. A cut-off level at 6 log CFU/g is chosen as the point of spoilage and development of off-flavours. R^2>90% for all fittings.

Walker (1998) reported that although yeasts are capable of growth over a wide pH range (2-8), their optimum pH for growth exists between 4 and 6, which was confirmed for *C. guilliermondii* in this study. Studies by Tokuoka (1993) and Narendranath and Power (2000) on *Zygosaccharomyces rouxii* and *Saccharomyces cerevisiae* respectively also revealed growth over wide pH levels and showed a similar tolerance for low pH by these species. Mechanisms of yeast adaptation to low pH mainly involve energy demanding systems that enable plasma membrane transporters to pump out the acid that penetrates into the cell (Devlieghere et al., 2013). Aside from the previously mentioned mechanism, some studies have shown that resistance to low pH may be connected to changes in the membrane conductivity to H^+ or possession of special gene expressions like those that code for protein kinase C in *S. cerevisiae*, which is required for cell integrity (Fernandes et al., 2005). Some yeasts also have cell wall sensors and metabolic pathways that are activated in response to acid stress (Almeida et al., 2014).

In YNB+SA systems at pH 3.5, growth of *C. guilliermondii* was completely inhibited with 100 mg/kg of sorbic acid at 7 °C and 22 °C. At pH 3.5, 98% of sorbic acid is in its undissociated form, which can freely diffuse through the cell membrane. The pH in emulsions is rarely sufficiently low to inhibit the growth of microorganisms by itself. In margarines without a dairy ingredient, the formulation can allow pH to be as low as 3.5. However, even this pH is not sufficient to limit the growth of yeasts and molds. Nevertheless, the pH value of the product is critical in many products, particularly when combined with preservatives (van Zijl and Klapwijk, 2000). From Figure 4.3 it can be seen that growth of *C. guilliermondii* was completely halted, even with a relatively low dose of 100 mg/L sorbic acid in the YNB+SA system at pH 4.5, at both 22 °C and 7 °C. As pH 4.5 is below the pKa value of sorbic acid (i.e. 4.76), most of the sorbic acid, i.e. 65%, will still be present in its undissociated form and exhibit inhibitory activity. Concentrations of sorbic acid needed for inhibition of bacterial growth range from 10 to 10000 mg/kg, with *Lactobacillus* sp. and *Clostridium* sp. being the most resistant (Lück, 1980). The effective concentration of potassium sorbate against bacterial growth in foods is usually between 500 and 3000 mg/kg, with the upper limit being established by problems with the taste of sorbate (Sofos and Busta, 1981).The GT in YNB+SA at pH 5.5 (Figure 4.4) was not significantly different than its counterpart without the preservative, YNB, at both 7 and 22 °C. The lag phase duration of YNB+SA curve at pH 5.5 was 10.6 ± 1.63 hours compared to 2.92 ± 0.98 hours for YNB at pH 5.5 at 22°C. Also, 6.41 ± 0.09 hours compared to 2.72 ± 1.0 hours was calculated for the YNB+SA and YNB curves, respectively, at pH 5.5 at 7 °C. Although the lag phases were longer at both temperatures for the YNB+SA systems compared to the YNB systems at pH 5.5, they were not significantly different ($p > 0.05$). The lack of significant differences in this case could be attributed to the fact that a large variation occurred in the

estimated lag phase durations throughout the 6 evaluated food model systems. The lag phase duration deviations could be a consequence of the inherent variability of biological material, where population cell division dynamics are dominated by the most robust cells in a population. This means that, in one subsample of a population, it would be these robust yeast cells that would drive cell division, while other, weak or damaged cells, would remain dormant for an extended period of time thus influencing the lag phase duration of the entire population. It is therefore generally observed that variations on lag phases are much more pronounced than on generation times. The GT and λ of YNB and YNB+SA at pH 6.5 at 22 °C were similar (Figure 4.5), indicating no effect of sorbic acid at this pH level. On the other hand, at 7 °C, the GT's of YNB and YNB+SA at pH 6.5 were significantly different. Adding low temperature as a hurdle slowed down yeast proliferation. Hurdle technology achieves synergies through the combined manipulation of physico-chemical conditions such as temperature, water activity, acidity and preservative concentration (Ohlsson and Bengtsson, 2002).

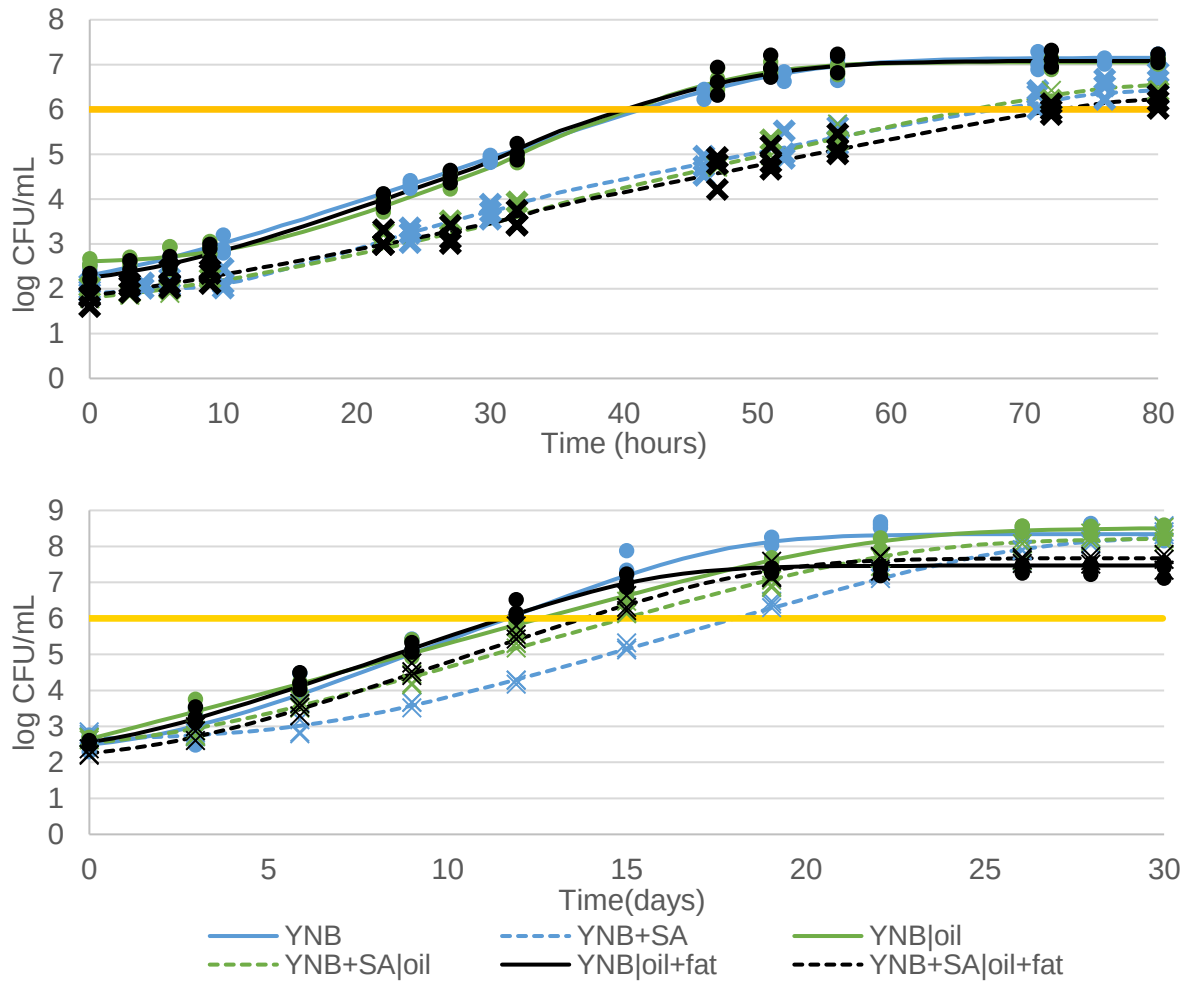


Figure 4.4. Dashed and full lines represent fitted growth curves of *C. guilliermondii* in YNB(SA), YNB(+SA)|oil and YNB(+SA)|oil+fat systems at pH 5.5 at 22 °C (top) and 7 °C (bottom). × and ● symbols represent sampling points from three replicates of systems with and without 100 mg/kg sorbic acid (SA) (i.e. 134 mg/kg potassium sorbate), respectively, as obtained by plate counts. A cut-off level at 6 log CFU/g is chosen as the point of spoilage and development of off-flavours. R^2>90% for all fittings.

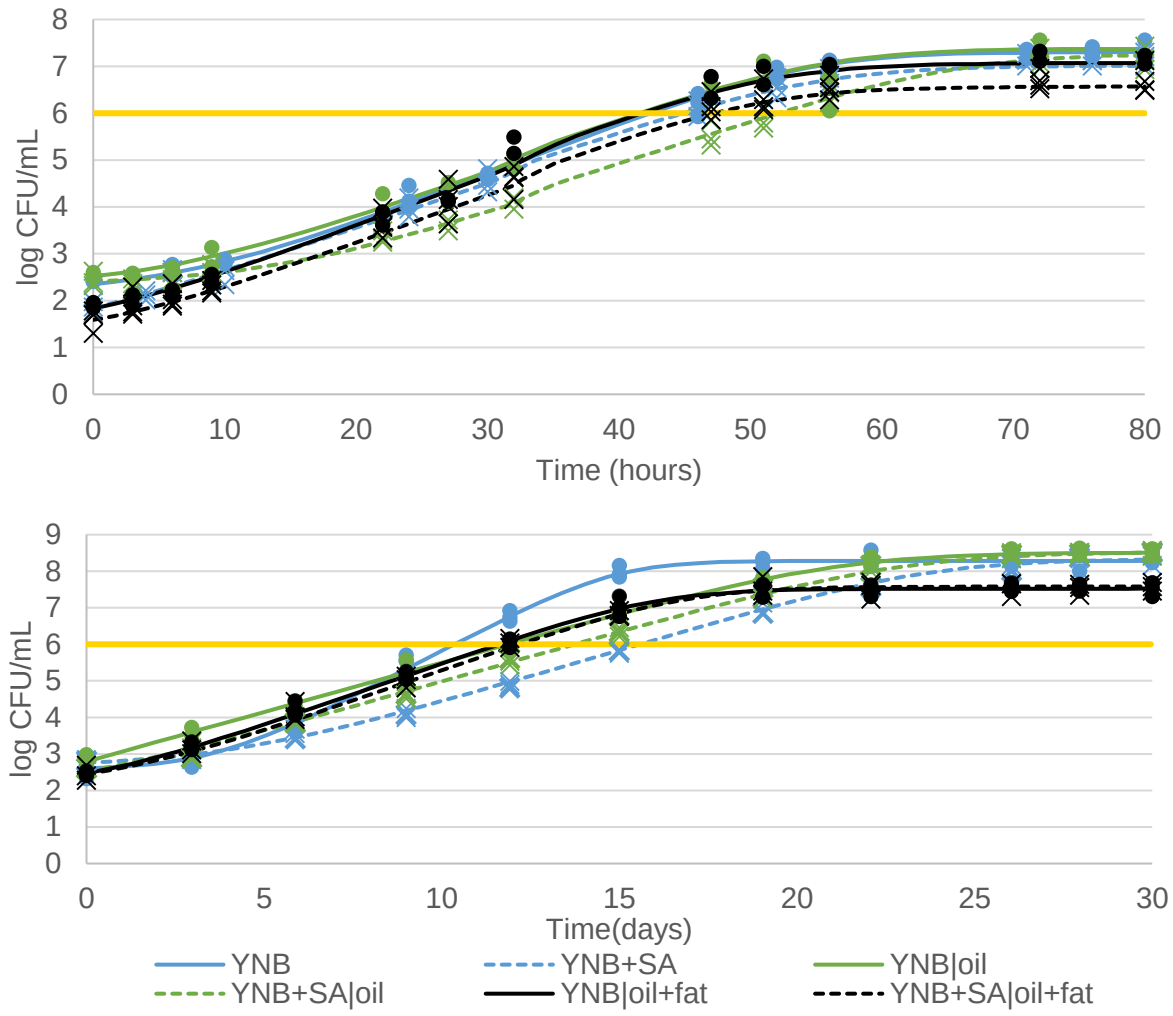


Figure 4.5. Dashed and full lines represent fitted growth curves of *C. guilliermondii* in YNB(+SA), YNB(+SA)|oil and YNB(+SA)|oil+fat systems at pH 6.5 at 22 °C (top) and 7 °C (bottom). × and ● symbols represent sampling points from three replicates of systems with and without 100 mg/kg sorbic acid (SA) (i.e. 134 mg/kg potassium sorbate), respectively, as obtained by plate counts. A cut-off level at 6 log CFU/g is chosen as the point of spoilage and development of off-flavours. R^2>90% for all fittings.

In order to make a more feasible and practical comparison between the different experimental conditions, a critical spoilage level was set at 6 log CFU/ml. This level was chosen because Ledenbach and Marshall (2009) stated that spoilage characteristics, such as yeast off flavours, changes in texture and bitter taste, manifest in butter when lipolytic yeasts grow to 6 log CFU/g. While at 7 °C, the yeast populations in YNB reach this spoilage level the fastest, this wasn't always the case for the populations at 22 °C. This was attributed to differences in inoculum size of ± 0.5 log CFU/mL for the experiments at 22 °C as they weren't initiated on the same days.

4.3.2. Influence of oil and solid fat on partitioning and effectiveness of sorbic acid

No significant differences between the GT's and lag phase durations of all YNB|oil curves of all pH values were observed ($p<0.05$) at both 7 °C and 22 °C (Table 4.1).

When comparing the GT's and lag phase durations of YNB and YNB|oil curves at 22 °C, both the GT's and lag phase durations of *C. guilliermondii* at pH 4.5, 5.5, 6.5 were not significantly different, while at pH 3.5, the addition of oil in the system shortened the lag phase and reduced the GT. At 7 °C the GT's and lag phase durations of YNB and YNB|oil curves at all pH's were significantly different than each other. Although significant differences were observed between these two unpreserved conditions, no definite direction of impact could be concluded.

The most interesting findings regarding the behavior of sorbic acid were observed with the inclusion of a lipid phase in the food model system. Adding a layer of liquid oil on top of the water phase induced partitioning of sorbic acid into the oil phase, reducing its inhibitory properties. At pH 3.5 at 22°C, adding oil on top of a preserved water phase in the YNB+SA|oil system, caused the reversal of the complete growth inhibition visible at the YNB+SA system. As there was still a substantial proportion of protonated sorbic acid present in the water phase of YNB+SA|oil system at pH 3.5, it had an influence on the growth parameters of *C.guilliermondii*, visible in the higher generation time and longer lag phase duration, compared to the YNB+SA|oil systems at pH 4.5, 5.5, 6.5. Interestingly, at 7 °C, the undissociated sorbic acid present in the YNB+SA|oil systems at pH 3.5 caused complete inhibition of growth of *C. guilliermondii.*

Since the purpose of the study was to quantify the difference in the growth parameters caused by addition of liquid oil and/or solid fat, as we had observed inhibition for systems at pH 3.5 at 7 °C in YNB+SA|oil, we omitted performing experiments with the conditions with solid fat. Firstly, this was done because we assumed that adding solid fat would completely inhibit the microorganisms, thus not permitting us to quantify growth parameters and second because of the laboriousness of the experimental design.

At pH 4.5 in YNB+SA|oil systems at 22 °C, the same phenomenon was observed as for pH 3.5. Adding an oil phase on top of the water phase induced partitioning of the dissociated sorbic acid into the lipid phase, thus reducing the aqueous phase sorbic acid concentration, $(HA)_{aq,eq}$, and allowing growth. However, at 7 °C, unlike YNB+SA|oil systems at pH 3.5 where growth was inhibited, growth at pH 4.5 occurred.

Table 4.1. Generation time (GT) and lag time (λ) duration of *C. guilliermondii* grown at different pH in YNB(+SA), YNB(+SA)|oil and YNB(+SA)|oil+fat systems at 22°C (hours) and 7°C (days). Means obtained from three replicates and ± denotes standard deviation. Within one column and one row, means with different textual and numerical superscripts, respectively, are significantly different at α=0.05. R^2>90% for all fittings.

22°C	**YNB**	**YNB+SA**	**YNB|oil**	**YNB+SA|oil**	**YNB|oil+fat**	**YNB+SA|oil+fat**
Generation time (Hours)						
pH 3.5	5.54 ± 0,70^{a1}	/	3.73 ± 0.17^{a2}	19.70 ± 3.63^{a3}	N/A	N/A
pH 4.5	3.63 ± 0.07^{b12}	/	3.86 ± 0.10^{a12}	4.27 ± 0.28^{b1}	3.41 ± 0.13^{a2}	4.31 ± 0.53^{a1}
pH 5.5	3.47 ± 0.04^{b1}	4.06 ± 0.05^{a12}	3.52 ± 0.16^{a1}	4.16 ± 0.49^{b2}	3.50 ± 0.09^{a1}	4.29 ± 0.07^{a2}
pH 6.5	3.51 ± 0.07^{b1}	3.53 ± 0.10^{b1}	3.41 ± 0.44^{a1}	3.62 ± 0.20^{b1}	3.46 ± 0.07^{a1}	3.57 ± 0.07^{b1}
Lag time (Hours)						
pH 3.5	10.23 ± 3.23^{a1}	∞	2.74 ± 1.91^{a2}	25.46 ± 1.40^{a3}	N/A	N/A
pH 4.5	2.84 ± 0.89^{b1}	∞	3.06 ± 2.76^{a1}	4.59 ± 3.05^{b1}	2.22 ± 1.25^{a1}	17.45 ± 3.07^{a2}
pH 5.5	2.92 ± 0.98^{b1}	10.6 ± 1.63^{a1}	5.65 ± 3.12^{a1}	6.47 ± 5.25^{b1}	2.85 ± 0.49^{b1}	3.22 ± 0.64^{b1}
pH 6.5	3.06 ± 1.31^{b1}	2.44 ± 1.96^{b1}	3.33 ± 1.35^{a1}	12.66 ± 2.20^{b2}	3.03 ± 2.85^{b1}	3.48 ± 2.27^{b1}
7°C						
Generation time (Days)						
pH 3.5	2.67 ± 0.95^{a1}	/	1.21 ± 0.04^{a2}	/	N/A	N/A
pH 4.5	0.66 ± 0.06^{b1}	/	1.15 ± 0.03^{a2}	1.17 ± 0.01^{a2}	0.75 ± 0.02^{a1}	/
pH 5.5	0.77 ± 0.23^{b1}	1.05 ± 0.02^{a12}	1.10 ± 0.08^{a2}	1.08 ± 0.01^{b12}	0.77 ± 0.13^{a12}	0.79 ± 0.08^{a12}
pH 6.5	0.58 ± 0.04^{b1}	1.05 ± 0.01^{a1}	1.11±0.04^{a2}	1.10 ± 0.04^{b2}	0.77 ± 0.05^{a3}	0.78 ± 0.03^{a3}
Lag time (Days)						
pH 3.5	3.87 ± 2.81^{a1}	∞	0.69 ± 0.54^{a2}	∞	N/A	N/A
pH 4.5	3.20 ± 0.51^{a1}	∞	1.42 ± 0.49^{a2}	10.86 ± 0.53^{a3}	2.02 ± 0.67^{a23}	∞
pH 5.5	2.72 ± 1.04^{a1}	6.41 ± 0.09^{a2}	0.81 ± 0.24^{a3}	2.54 ± 0.15^{b14}	1.19 ± 0.65^{a34}	2.04 ± 0.43^{a14}
pH 6.5	3.72 ± 0.56^{a1}	4.28 ± 0.35^{b1}	/	1.11 ± 0.53^{c2}	1.00 ± 0.52^{a2}	1.38 ± 0.65^{a2}

∞ denotes an extended lag phase, while / denotes no growth or no lag phase. N/A denotes data not available.

At pH 5.5 and 6.5, no significant differences between the GT's of YNB+SA and YNB+SA|oil systems were observed. Lag phase durations of the fore mentioned two systems were different, except at pH 5.5 at 22 °C, but no definite direction of impact could be concluded. At pH 5.5 and 6.5, the majority of sorbic acid is in its undissociated form, which cannot partition into the lipid phase and is retained in the water phase, where microorganisms are located.

Comparing YNB, YNB|oil and YNB|oil+fat systems at pH 4.5, 5.5, 6.5 at 22°C, it was observed that addition of solid fat didn't elicit a prominent change in neither the generation times nor lag phase durations of *C. guilliermondii.* At 7°C, although significant differences were observed between the GT's and λ's of the forementioned systems, these differences were not systematically present throughout all pH values, thus we couldn't conclude that there is strong evidence that adding a lipid phase greatly influences growth of *C. guilliermondii* in a non-preserved system.

The key finding of this study was observed upon adding solid fat as a constituent of the lipid phase onto the water phases that contained sorbic acid. It was demonstrated that solid fat influences water phase sorbic acid concentrations. The reasons for this effect are two-fold: (i) the presence of solid fat in the lipid phase indicates that there is less liquid oil available for the protonated sorbic acid to partition into and (ii) diffusion of sorbic acid into the lipid phase is retarded when solid fat is present. Best visible in food model systems at pH 4.5, the addition of fat in the YNB+SA|oil+fat system hinders sorbic acid partitioning in comparison to the results obtained from the YNB+SA|oil system. At 22 °C, the lag phase duration of YNB+SA|oil+fat system at pH 4.5 was significantly prolonged compared to the YNB+SA|oil system and the critical spoilage value of 6 log CFU/mL wasn't reached after 80 hours. The GT wasn't significantly different between the two fore mentioned curves. Nevertheless, partitioning of the acid occurred, shown by a reduction of the acid's inhibitory activity and eventual growth of *C. guilliermondii* compared to the complete inhibition occurring in the YNB+SA system. Interestingly, at 7 °C, the presence of solid fat and 100 mg/kg sorbic acid completely inhibited growth of the microorganism in the YNB+SA|oil+fat system at pH 4.5, whereas growth still occurred in YNB+SA|oil.

HPLC analysis was performed to determine $((HA)_{aq,eq}+(A^-)_{aq,eq})$ in YNB+SA|oil and YNB+SA|oil+fat systems. The results are summarized in Table 4.2. Measured values are results of HPLC analysis, while the expected values have been calculated with the aid of the sorbic acid distribution model (Chapter 2), taking into account the pH, ratio of oil and measured SFC (Paragraph 4.2.8.). The measured valued are all higher than the expected ones, possibly

due to the time delay in partitioning. Figure 4.6 shows the development of sorbic acid partitioning during 168 hours at 22°C and during 1 month at 7°C. At 22°C, the analysis period was longer than microbiological testing to better observe the time dependent partitioning.

Table 4.2: $((HA)_{aq,eq}+(A^-)_{aq,eq})$ / (mg/kg) in model YNB+SA|oil and YNB+SA|oil+fat systems at 22 °C and 7 °C. Means obtained from three replicates, injection performed in duplicate, ± denotes standard deviation.

		Measured		Expected	
		7°C	22°C	7°C	22°C
pH 4.5	Oil	34.1 ± 0,4	43.6 ± 1.1	22	22
	Fat	87.0 ± 2.0	87.0 ± 1.0	30	26
pH 5.5	Oil	74.9 ± 2.8	86.0 ± 0.4	54	54
	Fat	101.3 ± 1.1	101.0 ± 0.6	64	60
pH 6.5	Oil	99.0 ± 1.0	103.1 ± 0.0	90	90
	Fat	102 ± 0.1	105.0 ± 1.4	93	92

Since there was a large proportion of undissociated acid present at pH 4.5, the majority of it was able to gradually diffuse into oil. When we compared results of the microbiological analysis to HPLC results, the evidence of different partitioning behaviour between liquid oil and solid fat systems was reaffirmed. Namely, the onset of growth in the YNB+SA|oil system at pH 4.5 at 22 °C happened at 4.59 ± 3.05 hours, when the acid already started partitioning and its active aqueous phase concentration, $(HA)_{aq,eq}$, decreased. After 168 hours, there was 44 mg/kg of $((HA)_{aq,eq}+(A^-)_{aq,eq})$ present in the water phase of the YNB+SA|oil system at pH 4.5 at 22°C. At 7°C, the same partitioning behaviour was observed, albeit the average final concentration after one month was 34 mg/kg, lower than the final concentration measured at 22°C. The phenomenon of partitioning also occured in the YNB+SA|oil+fat system at pH 4.5, resulting in 87 mg/kg of $((HA)_{aq,eq}+(A^-)_{aq,eq})$ at the end of trial period at 22 °C. The spoilage level of 6 log CFU/mL was not reached after 80 hours, since the solid fat delayed sorbic acid transition into the lipid phase, thus leaving more sorbic acid in the water phase, making it available to retard growth.

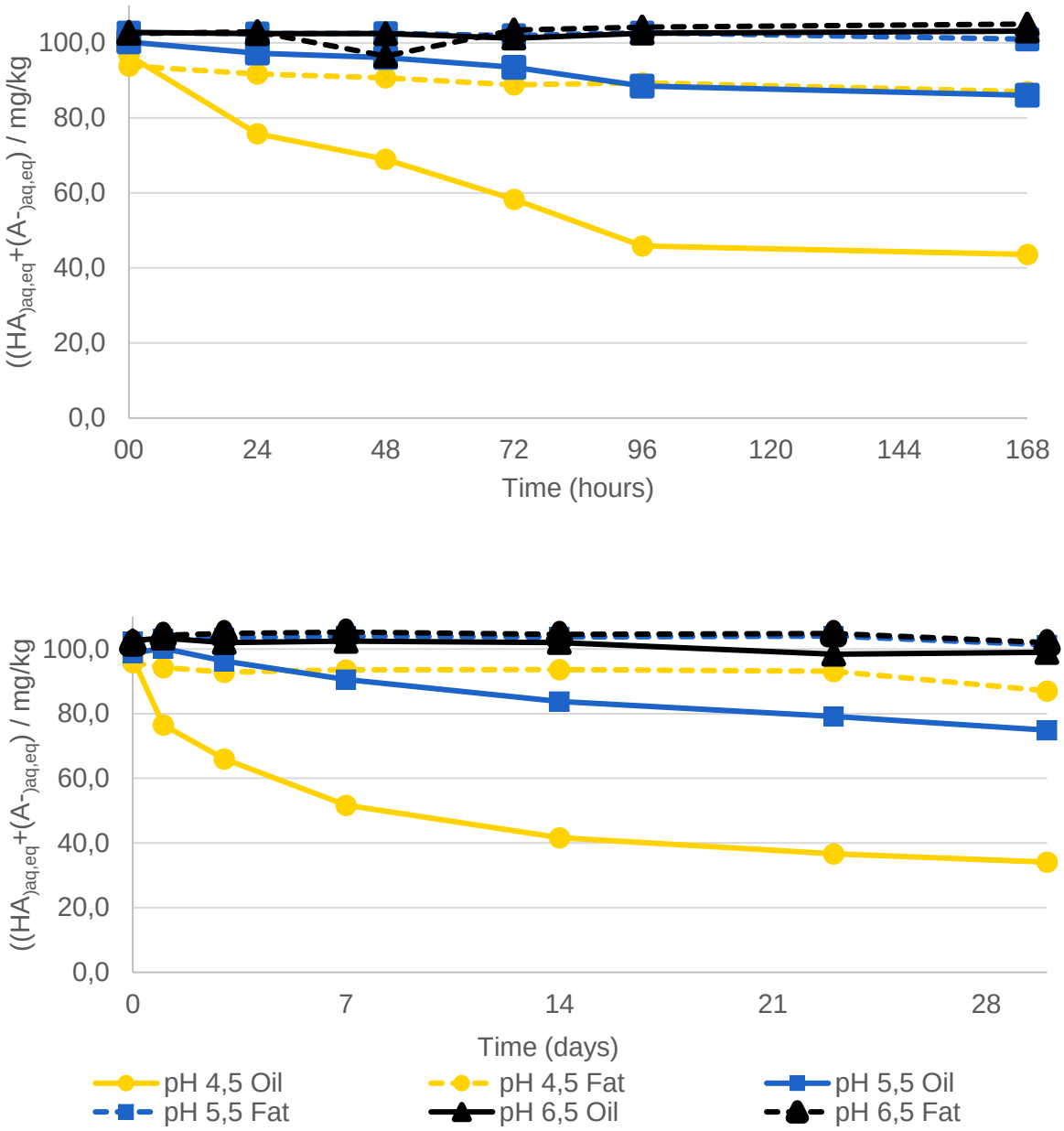


Figure 4.6. $((HA)_{aq,eq}+(A^-)_{aq,eq})$ / (mg/kg) in water phase of model YNB+SA|oil (full lines) and YNB+SA|oil+fat (dashed lines) systems at 22 °C (top) and 7 °C (bottom). ●, ■ and ▲ represent systems at pH 4.5, 5.5, 6.5, respectively. Means obtained from three replicates, injection performed in duplicate.

In the YNB+SA|oil+fat at pH 4.5 at 7 °C, the measured final concentration of preservative was the same as at 22 °C, 87 mg/kg $((HA)_{aq,eq}+(A^-)_{aq,eq})$ on average. This relatively large water phase sorbic acid concentration could be explained by the assumption that the presence of solid fat retards diffusion of sorbic acid into the lipid phase but also that there is less liquid oil available for sorbic acid to partition into, comparing to the system with only liquid oil. This observation also prompted an assumption that the solid fat might not able to dissolve sorbic acid as it is present in the crystalline form. GT's of *C. guilliermondii* in samples at pH 5.5 kept at 22 °C were similar in YNB+SA, YNB+SA|oil and YNB+SA|oil+fat systems. In YNB+SA|oil

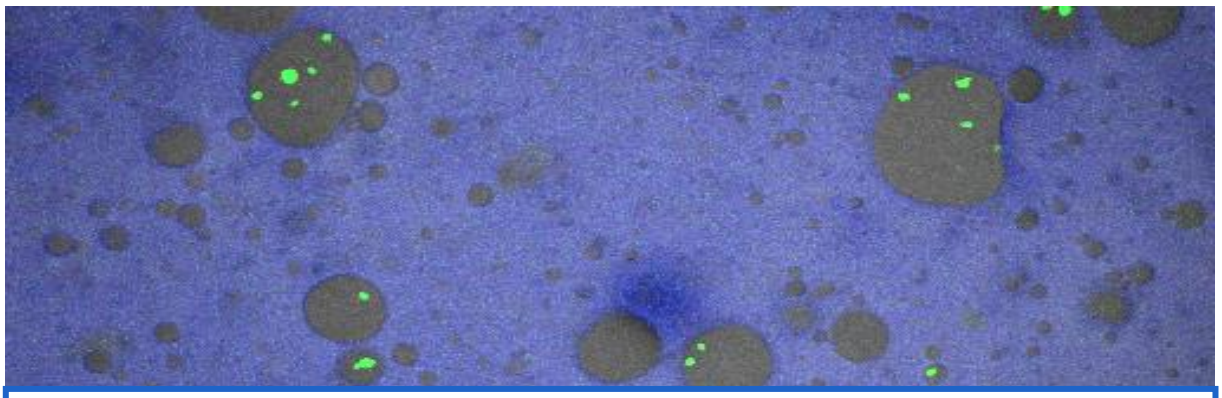
systems at pH 5.5 at 7 °C and 22 °C the concentration of ($(HA)_{aq,eq}$+$(A^-)_{aq,eq}$) was on average 75 and 86 mg/kg, respectively. At pH 5.5, there was a larger proportion of dissociated acid present than at pH 4.5, thus ($(HA)_{aq,eq}$+$(A^-)_{aq,eq}$) was larger since a smaller proportion of sorbic acid was able to partition into the lipid phase. In YNB+SA|oil+fat systems at pH 5.5 at both temperatures, there was no evidence of partitioning. In both YNB+SA|oil and YNB+SA|oil+fat systems at pH 6.5, ($(HA)_{aq,eq}$+$(A^-)_{aq,eq}$) concentrations remains unchanged, due to most of the acid being present in dissociated form and not being able to partition into the non-polar lipid phase. No prominent effect of the addition of solid fat on the GT was observed for YNB+SA|oil+fat at pH 6.5 in samples kept at 22 °C. However, both pH and the presence of oil and fat were determined to have significant effects on the antimicrobial activity of sorbic acid towards *C. guilliermondii.*

CONCLUSION

Albeit scarce, information about behaviour of preservatives in water/liquid oil systems does exist. This information mentions pH and the ratio of oil to water as factors determining the concentration of active protonated sorbic acid in the aqueous phase. Information on other factors such as type and amount of fat, droplet size and pH influencing partitioning of preservatives in biphasic systems can be found, but the statements are made in the scope of pharmaceuticals and we haven't found evidence of experimental validation for the statement about the type and amount of fat influencing preservative behaviour. To our best knowledge, this investigation on the influence of solid fat on the partitioning of sorbic acid in a food model system is the first of its kind.

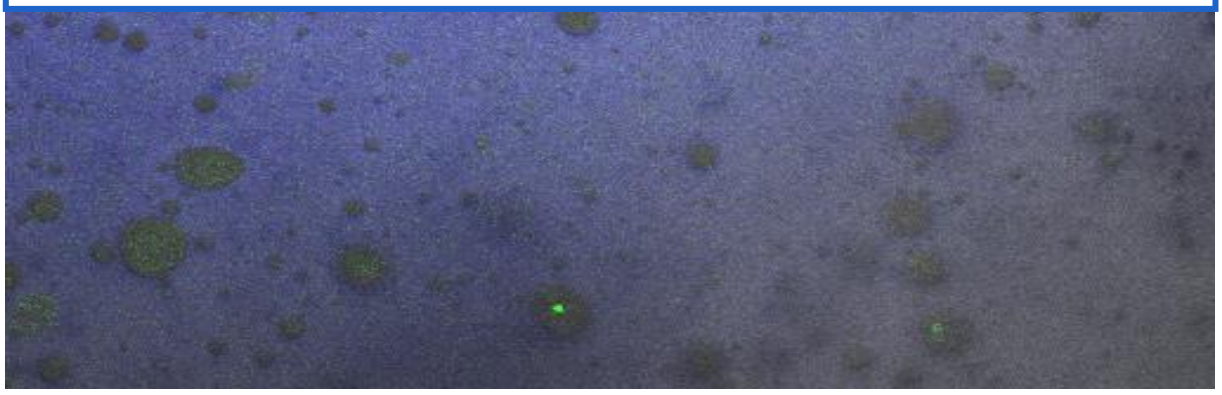
Our investigation confirms that sorbic acid partitioning is dependent on the type of fat, where solid fat retards the diffusion of this carboxylic acid into the lipid phase. The presence of solid fat also reduces the amount of liquid oil available in the lipid phase, making less oil available for partitioning, thus increasing the aqueous phase concentration of sorbic acid. Also, in line with previous findings, our study also confirms that pH is a key factor in preservative distribution in biphasic systems comprised of a water and lipid phase.

This information may be valuable to emulsion producing facilities, whether they are oriented to food, cosmetics or pharmaceuticals. By controlling the SFC of the lipid phases in their recipes, producers can influence retention of the preservative in the water phase, thus reducing spoilage potential and ensuring safer products.



CHAPTER 5

Influence of temperature and emulsion water droplet size distribution (DSD) on growth of *C. guilliermondii* in recombined butter



SUMMARY

The ability of microorganisms to grow in W|O emulsions is determined by the intrinsic properties of the aqueous phase such as the water droplet size distribution, nutrient content, the presence of growth inhibiting components and the keeping temperature of the product.

The aim of this study was to investigate the influence of temperature (7 °C and 22 °C) and emulsion water droplet size distribution (DSD) on the growth of *Candida guilliermondii,* in recombined butter made with (82 and 61%) anhydrous milk fat (AMF) during 21 days of storage. Prior to this, different DSDs were achieved by varying Ultra Turrax rotation speed (5000-15000 rpm) during emulsification.

The proportion of potentially microbiologically vulnerable water droplets (diameter > 10 µm) was larger in samples emulsified at 5000 rpm than at 15000 rpm in both 82 and 61% AMF recombined butter. Smaller, microbiologically stabile droplets were more likely to be present in 82% AMF recombined butter, irrespective of Ultra Turrax rotation speed. There was no change in the proportion of potentially vulnerable water droplets during 21 days of storage at 7 °C, in both inoculated 82 and 61% AMF recombined butter, indicating that refrigeration aids product stability. Growth of *C. guilliermondii* was observed in 61% AMF butter at 7 °C which was not the case in the 82% AMF butter, indicating that the proportion of vulnerable droplets at the time of production could also play a role in product stability. At 22 °C, the proportion of potentially vulnerable droplets increased during 21 days of storage in both inoculated 82 and 61% AMF recombined butter by approximately 20%, indicating emulsion destabilization. Microbial counts of *C. guilliermondii* increased at 22 °C, especially in 61% AMF recombined butter, which could have been a consequence of emulsion destabilization in combination with the elevated keeping temperature.

The results of this study could be of interest to researchers investigating fungal behavior in W|O emulsions and also to emulsion producing facilities in need of a laboratory scale emulsification method allowing for microbiological challenge testing.

5.1. Introduction

According to EC Regulation 98/582 recombined butter is defined as a product obtained from anhydrous milk, non-fat dry milk extract and water. Anhydrous milkfat, variously called AMF, anhydrous butteroil, anhydrous butterfat, butterfat or butteroil, is the product exclusively obtained from milk, cream or butter by removing almost all traces of moisture and solids-not-fat material (Illingworth and Bissell, 1994). Although W|O emulsions, such as butter and margarine, are not considered to be highly perishable foods, they are known to undergo bacterial and fungal spoilage (ICMSF, 2005; Meshref, 2010).

In this study, varying DSD in non-inoculated recombined butter were first achieved by varying Ultra Turrax rotation speed during emulsification. Then, after inoculation of the water phase with 2 log CFU/g *C. guilliermondii* , 82 and 61% AMF recombined butters were emulsified at 10000 rpm , Inoculated recombined butters were then placed at 7 °C and 22 °C and analyzed for growth during 21 days. pfg-NMR measurements were performed in parallel to obtain the DSD and D_{43} of the inoculated recombined butters as a function of storage time.

The goals of the study were to investigate the influence of (i) Ultra Turrax rotation speed on the DSD, the influence of (ii) temperature and (iii) DSD on the growth of *C. guilliermondii* in self-made recombined butter. This study also elucidates a method of laboratory scale W|O emulsion making, which is useful to emulsion making facilities unable to perform challenge testing in the production line due to contamination hazards.

5.2. Materials and methods

5.2.1. Yeast strain and culturing conditions

For pre-culturing conditions of *C. guilliermondii* and media composition, refer to Paragraph 4.2.1. Two days before the preparation of the inoculated recombined butter, one *C. guilliermondii* colony was taken from YNA plates, placed into 10 mL YNB and incubated for 48 hours at 30 °C with the aid of a shaker set at 150 rpm. Shaking increased the final yield of *C. guilliermondii* from 7 log CFU/mL to 8 log CFU/mL. Inoculation of 10 mL YNB was done in triplicate to ensure enough inoculum for challenge testing in recombined butter.

5.2.2. Preparation of aqueous phase of recombined butter

In experiments investigating the influence of Ultra Turrax rotation speed on DSD, the aqueous phase was solely distilled water. In experiments investigating the influence of temperature and DSD on the growth of *C. guilliermondii,* the aqueous phase was prepared as follows: YNB (1 mL) with 8 log CFU/mL *C. guilliermondii,* described in Paragraph 5.2.1., was taken and placed into 2 mL Eppendorf tubes (Eppendorf, Germany). The Eppendorf tubes were then centrifuged for 10 minutes at 10000 rpm in Sorvall™ Legend™ Micro 17 Centrifuge (ThermoFisher Scientific, USA). The supernatant was removed and the pellet was resuspended in 1 mL of sterile YNB. The rest of the volume to make up the aqueous phase was sterile distilled water. Dilution series were prepared in PPS to obtain an approximate inoculum level of 2 log CFU/g *C. guilliermondii* in 400 g of recombined butter. In 61% AMF recombined butter, 1.5% (on total product mass) of starch (Corman SA, Goe, Belgium) was added into sterile distilled water and mixed with a magnetic stirrer at 700 rpm 2 hours before butter making.

5.2.3. Preparation of lipid phase of recombined butter

AMF (99.9%) (Corman SA) was used for the preparation of the lipid phase of recombined butter. Solid AMF was placed in a 1 L glass beaker (Schott, Germany), melted in the microwave, returned to a closed glass recipient and placed in a water bath at 48 °C to remain liquid. 0.5% (on total product mass) of PGPR (Corman SA) was added to the bulk lipid phase 1 hour before butter making. The mixture of AMF and PGPR was homogenized with the Ultra Turrax for one minute at 10000 rpm to allow for complete mixing of the two components.

5.2.4. Influence of Ultra Turrax rotation speed on DSD and D_{43}

In order to obtain recombined butter with varying DSD and D_{43}, different Ultra Turrax rotation speeds (5000-15000 rpm) were applied during emulsification (See Paragraph 5.2.5) and pfg-NMR (See Paragraph 5.2.7) analysis was performed on the prepared emulsions.

5.2.5. Preparation of inoculated recombined butter

82% AMF recombined butter was made by mixing 82% AMF, 17.5% aqueous phase and 0.5% PGPR on total mass of product. 61% AMF recombined butter was made by mixing 61% AMF, 37% aqueous phase, 1.5% starch and 0.5% PGPR on total mass of product.

The aqueous phase (as described in Paragraph 5.2.2) was placed into the hot water bath at 48 °C for maximum 10 minutes before butter making. The temperature of the aqueous phase and lipid phase before mixing needs to be above the melting point of the lipid phase. (Kirkeby, 2006). However, due to the possibility of further microbial proliferation in the inoculated aqueous phase at elevated temperature, the time in the water bath needed to be limited. The aqueous and lipid phase were emulsified at 10000 rpm for three minutes with the aid of an ethanol sterilized Ultra-Turrax homogenizer (IKA T25 Digital, Germany). After emulsification, the liquid emulsion was transferred into an ethanol sterilized cooling bowl which was previously kept at -18 °C for at least 24 hours. The cooling bowl is a part of the benchtop semi-professional frozen dessert maker (Cooking Chef KM070, Kenwood). Cooling and crystallization was performed for 3 minutes at a constant low speed setting (3) and the emulsion was continuously scraped of the walls of the cooling bowl with the aid of inbuilt plastic scrapers. Batches of recombined butter were then placed in a sterile stomacher bag, sealed and left at 4 °C for 4 hours to complete the crystallization process (Lund et al., 2000). Afterwards, the recombined butter was portioned into 10 ± 2 g portions in sterile stomacher bags and placed at 7 °C and 22 °C. One portion from each batch (triplicates) was foreseen for each microbiological sampling time.

5.2.6. Sampling procedure for recombined butter

The ISO 6887-5:2010 sampling procedure was followed which recommends weighing an analytical unit of 10 g in a sterile recipient, transferring to a temperature-controlled hot water bath at 45°C, for no longer than 10 minutes, until the sample is completely melted. Then, 90 mL of buffered peptone water (BPW) (Oxoid, United Kingdom), warmed at 45 °C, was added to the sample and mixed. Sampling was performed on day 0,1,2,3,4,7,10,14,17 and 21 of trial period.

5.2.7. Pulsed field gradient nuclear magnetic resonance (pfg-NMR) measurements

DSD and D_{43} were measured using a Maran Ultra 23 MHz pfg-NMR Analyzer (Oxford Instruments, UK). pfg-NMR test tubes (1 cm diameter) with a flat bottom were filled with recombined butter until the mark and placed at 5 °C in the water bath of the pfg-NMR machine one hour prior to analysis.

Calibration of the pfg-NMR machine was performed with AMF and distilled water. RINMR software V5.0.0.0. was used for calculations regarding the calibration of the pfg-NMR machine. *droplet* software V1.2.1.99. was used for integration and final calculation of both DSD and D_{43}.

pfg-NMR analysis was performed on the same days as microbiological analysis, as described in Paragraph 5.2.6.

5.2.8. Confocal laser scanning microscopy (CLSM)

The structure of an inoculated 82% AMF recombined butter sample was examined using a Nikon A1R CLSM microscope (Nikon Inc., Melville, NY, USA). Approximately 50 µl of 2 µg/mL solution of Nile Red (Sigma Aldrich, S. Louis, MO, USA) was mixed with the lipid phase of the recombined butter before butter making. A 488 nm Ar laser was used for excitation. GFP emission was detected between 505 nm – 525 nm and Nile Red emission between 710 nm - 850 nm.

5.2.9. Statistical analysis

Minitab® 17.1.0 was used to compare D_{43} among the different factors involved in this study and a 5% significance level was applied for all statistical tests. Normality was tested over normal probability plots. Homoscedasticity was tested with F-tests. One-way ANOVA in conjuction with Tukey's multiple comparisons post-hoc test were performed to compare sample means and establish if significant differences occurred.

5.3. Results and discussion

5.3.1. Influence of Ultra Turrax rotation speed on DSD and D_{43} in recombined butter

Chemical, pharmaceutical, specialty foods and biotechnology facilities all use high-speed homogenizers to emulsify, disperse, mix and process their products (Floury et al., 2004). Many of the most important properties of emulsion-based food products (e.g. shelf life, appearance, texture and flavor) are determined by the DSD of the emulsion (McClements, 2015). When presenting DSD data, it is convenient to divide the size range of droplets into a number of discrete size classes and stipulate the number or percentage of droplets that fall into each class (Hunter, 1986). The discrete size classes used in our study for the classification of water droplet size were: (i) <2 µm, (ii) 2-5 µm, (iii) 5-10 µm, (iv) 10-15 µm, (v) 15-30 µm, (vi) >30 µm. Figure 5.1 and 5.2 depict selected cumulative and probability DSD curves in 82 and 61% AMF recombined butter made with varying Ultra Turrax rotation speed, respectively. Only DSD curves obtained from recombined butter emulsified at 5000, 10000 and 15000 rpm are depicted to ease data visualization.

(A) **(B)**

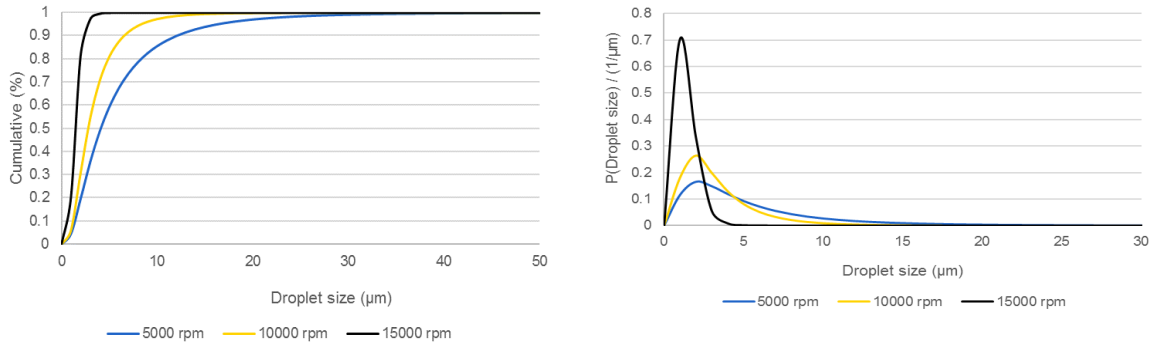


Figure 5.1. Selected cumulative (A) and differential (B) DSD curves in 82% AMF recombined butter made by varying Ultra Turrax rotation speed.

(A) **(B)**

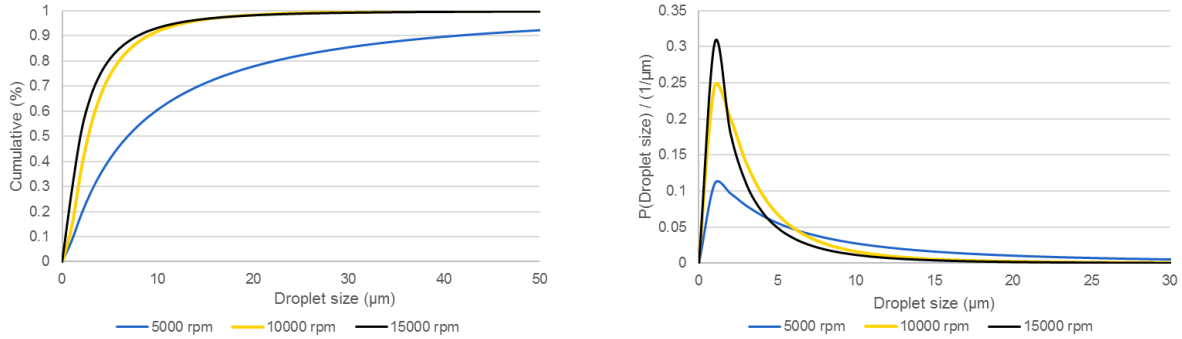


Figure 5.2. Selected cumulative (A) and differential (B) DSD curves in 61% AMF recombined butter made at varying Ultra Turrax rotation speed.

The cumulative distribution function calculates the cumulative probability for a given droplet size. The probability density (differential) function helps identify regions of higher and lower probabilities for the occurrence of a water droplet of a given size.

The slope of the cumulative DSD curves in Figure 5.1.(A) is steeper than in Figure 5.2.(A), indicating that the water droplets in 82% AMF recombined butter were smaller than those in 61% AMF recombined butter, irrespective of the rotation speed used during emulsification. Consequentially, the probability of occurrence of a smaller water droplet is higher in 82% AMF recombined butter (Figure 5.1.(B)), than in 61% AMF recombined butter (Figure 5.2.(B)), irrespective of the rotation speed used during emulsification.

Droplet size in emulsions usually decreases as emulsification time or the rotation speed of the stirrer is increased, until a lower limit is achieved which depends on the nature and concentration of the ingredients used. Typically, the droplets produced by a high-speed blender range between about 2 and 10 µm in diameter (McClements, 2015). In our study, it was also observed that droplet size decreased as rotation speed of the Ultra Turrax increased. Our findings were also in line with the conclusions from Lyoo et al. (2005) where it was found

that as agitation speed of the homogenizer increased from 5000 to 20000 rpm, so did the droplet size distribution in 20% oil W|O emulsions shift to small diameters.

Figure 5.3 shows the influence of varying the Ultra Turrax rotation speed on D_{43} in 82 and 61% AMF recombined butter where rotation speed was varied in 1000 rpm increments from 5000 to 15000 rpm.

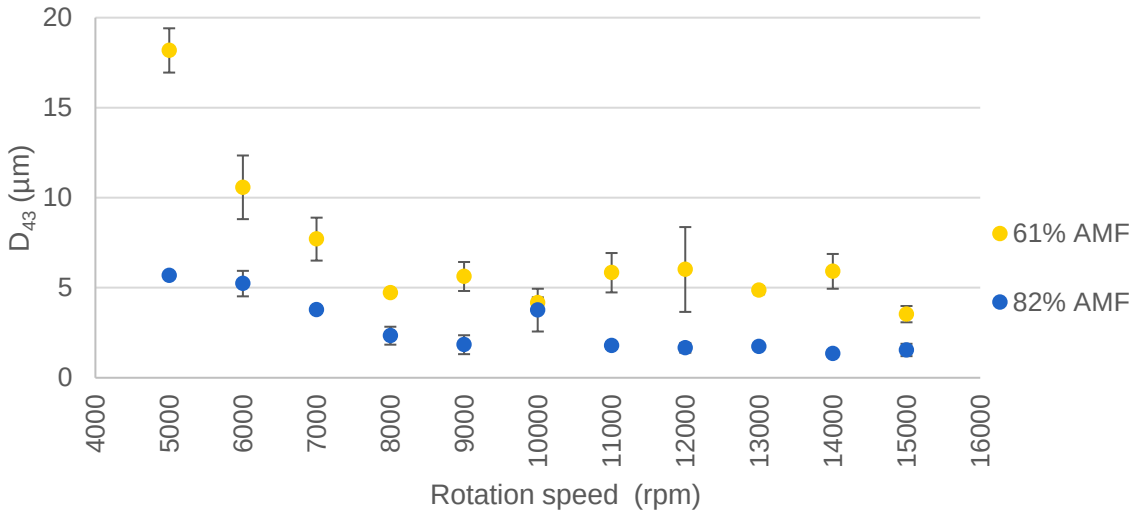


Figure 5.3. Effect of Ultra Turrax rotation speed on D_{43} (µm) in 82 and 61% AMF recombined butter. Averages taken from three replicates. Error bars denote standard deviation.

Pajouhandeh et al. (2016) reported that photomicrographs revealed the D_{43} in emulsions increased by enhancing the aqueous phase fraction from 5 to 20% on total emulsion weight. In our study, increasing the aqueous phase fraction (from 17.5% in 82% AMF recombined butter to 37% in 61% AMF recombined butter) also resulted in higher D_{43}, irrespective of the Ultra Turrax rotation speed used during emulsification. The largest differences in D_{43} could be observed between 82 and 61% AMF recombined butter emulsified at 5000 rpm.

5.3.2. DSD in 82 and 61% AMF inoculated recombined butter

Balinov et al. (1994) have successfully validated the use of nuclear magnetic resonance for determinations of water droplet sizes in margarine and low-calorie spreads (40 to 80% fat).

Figures 5.4 and 5.5 depict selected cumulative and probability DSD curves in 82% AMF recombined butter inoculated with 2 log CFU/g *C. guilliermondii* during 21 days of storage at 7 and 22 °C, respectively. Only DSD curves obtained from recombined butter at Day 0, 14 and 21 are depicted to ease data visualization.

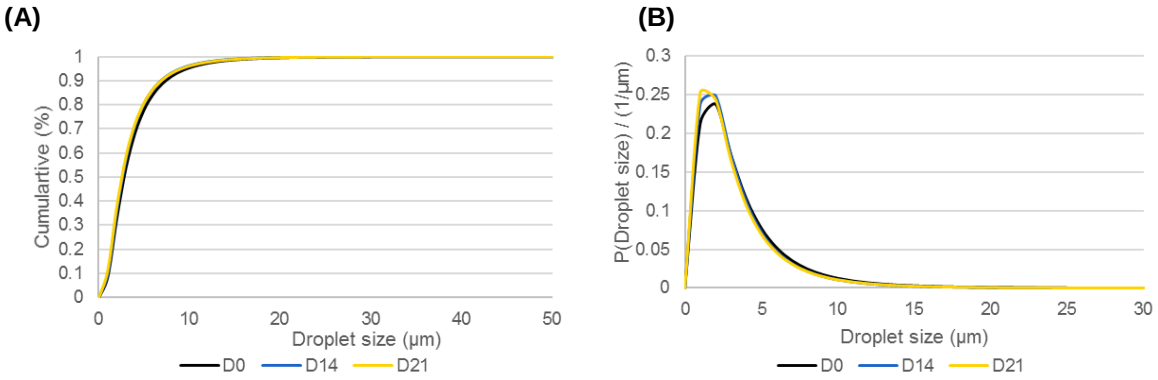


Figure 5.4. Selected cumulative (A) and differential (B) DSD curves in 82% AMF recombined butter inoculated with 2 log *C. guilliermondii* and stored at 7 °C for 21 days. D0, D14 and D21 signify days 0, 14 and 21 of trial period, respectively.

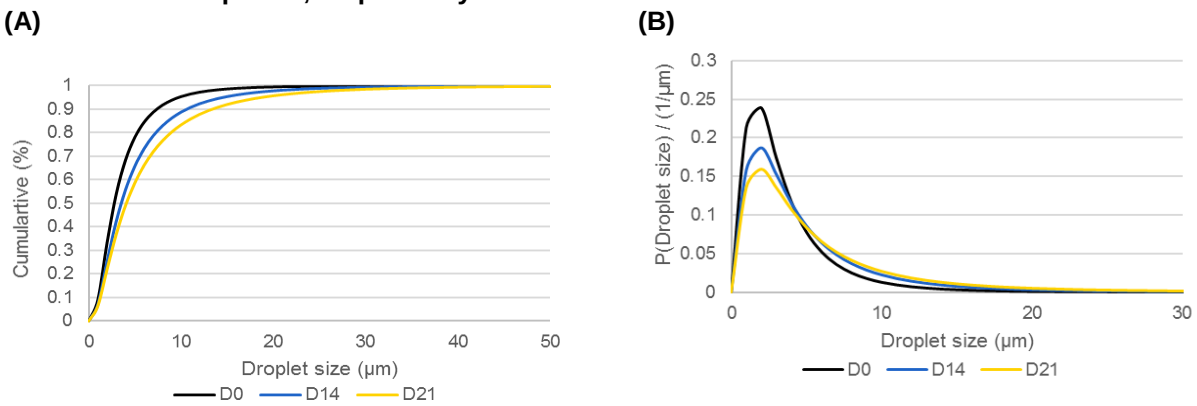


Figure 5.5. Selected cumulative (A) and differential (B) DSD curves in 82% AMF recombined butter inoculated with 2 log *C. guilliermondii* and stored at 22 °C for 21 days. D0, D14 and D21 signify days 0, 14 and 21 of trial period, respectively.

The water phase in W│O emulsions is generally present as dispersed droplets that are in a range of between 1 and 30 µm in diameter (Charteris, 1995). McClements (2015) stated that in butter, water droplets should preferably not exceed 10 µm in diameter. Boysen (1927) concluded that moisture droplets of less than 10 µm in diameter are too small to permit microbial growth. In 2001, ter Steeg et al. reported that the size of a water droplet has to exceed an estimated minimal value to allow for fungal germination, and stipulated a minimum diameter of 6.5 µm. Due to the lack of the information for a minimum diameter allowing for proliferation of yeasts and the fact that the latter mentioned diameter value is a fitted, rather than an experimental value, it was decided to use 10 µm as the diameter that would classify a water droplet as microbiologically vulnerable, to ease interpretation of the data.

The proportion of microbiologically vulnerable droplets stayed similar (ca 5%) during storage time in 82% AMF recombined butter at 7 °C (Figure 5.4.(A)). On the other hand, the proportion of vulnerable droplets increased from 5 to 17% during 21 days of storage in 82% AMF

recombined butter at 22 °C (Figure 5.5.(A)). Consequentially, the probability of occurrence of a microbiologically stabile (< 10 µm in diameter) water droplet reduced during storage at ambient temperature (Figure 5.4.(B) and 5.5.(B)). Generally, elevated temperatures result in destabilization, while reduced temperatures maintain emulsion stability (Touitou, 1998).

Figures 5.6 and 5.7 depict selected cumulative and probability DSD curves in 61% AMF recombined butter inoculated with 2 log CFU/g *C. guilliermondii* during 21 days of storage at 7 and 22 °C, respectively.

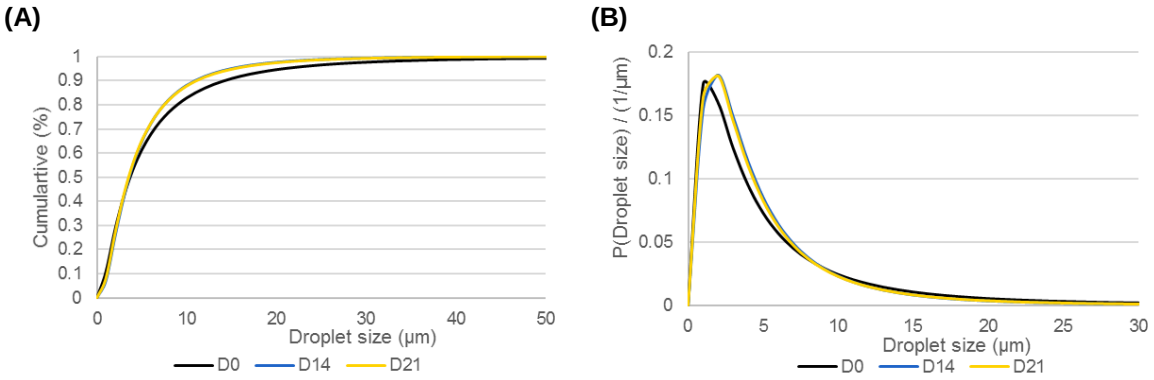


Figure 5.6. Selected cumulative (A) and differential (B) DSD curves in 61% AMF recombined butter inoculated with 2 log *C. guilliermondii* and stored at 7 °C for 21 days. D0, D14 and D21 signify days 0, 14 and 21 of trial period, respectively.

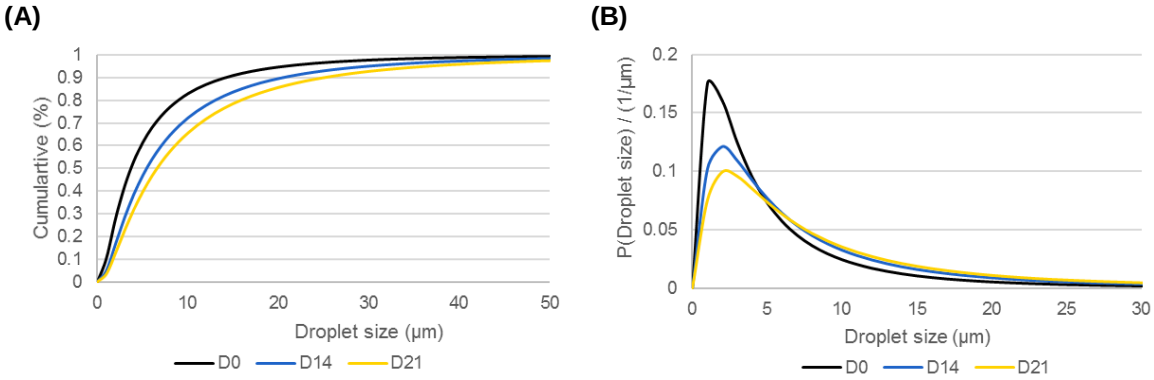


Figure 5.7. Selected cumulative (A) and differential (B) DSD curves in 61% AMF recombined butter inoculated with 2 log *C. guilliermondii* and stored at 22 °C for 21 days. D0, D14 and D21 signify days 0, 14 and 21 of trial period, respectively.

As was noticed in 82% AMF recombined butter, the proportion of microbiologically vulnerable droplets stayed similar during time in 61% AMF recombined butter at 7 °C (Figure 5.6.(A)). The proportion of vulnerable droplets increased from 18 to 35% during 21 days of storage in 61% AMF recombined butter at 22 °C (Figure 5.7.(A)). Stabile droplets were more likely to occur in 82% (Figure 5.4.(B) and 5.5.(B)) than at 61% (Figure 5.6.(B) and 5.7.(B)) AMF recombined butter, irrespective of storage temperature.

Rousseau et al. (2009) investigated the influence of temperature on the destabilization of the water droplets in butter and margarine by comparing and following changes in droplet size (D_{43}), solid fat content (SFC) and fat crystal spatial organization in the 28–34 °C range. They observed that during 96 h of storage at 28 °C, both butter and margarine were stabile, with similar D_{43} values (approximately 6 µm) and DSDs. As the storage temperature was raised above 30 °C, notable destabilization of the dispersed phase was observed. In this study, the recombined butter destabilized at 22 °C, but the time of exposure to elevated temperature was notably longer.

5.3.3. D_{43} in 82 and 61% AMF inoculated recombined butter

The volume weighted mean diameter of an emulsion (De Brouckere mean diameter; D_{43}) is the weighted average volume diameter, assuming spherical water droplets of the same volume as the actual droplets. It reflects the size of those water droplets which constitute the bulk of the sample volume. It is most sensitive to the presence of large droplets in the size distribution (Piacentini, 2016). Figure 5.8 shows the D_{43} measured in recombined butter as described in Paragraph 5.2.5.

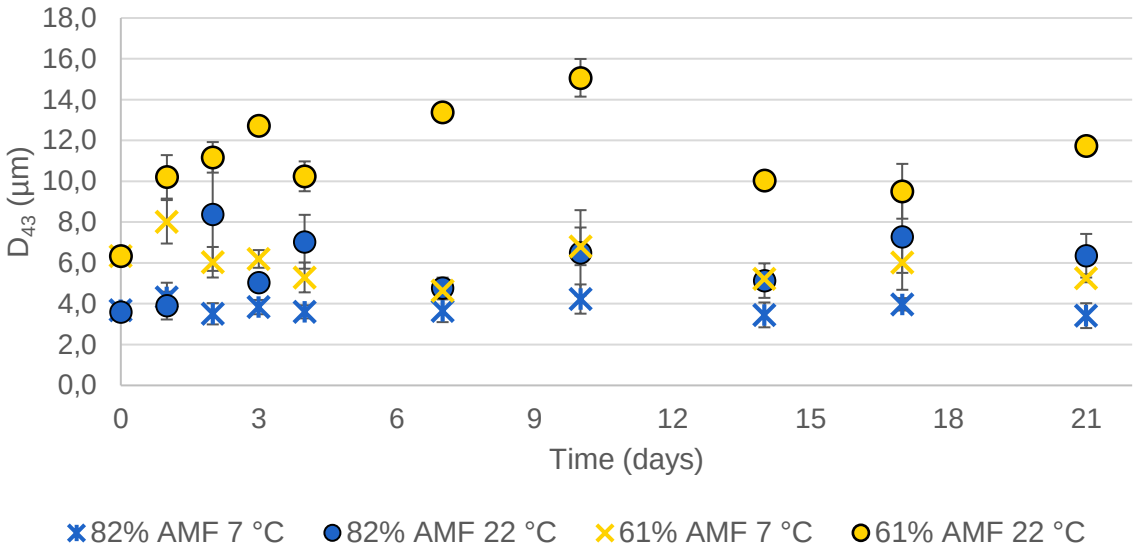


Figure 5.8. D_{43} (µm) in 82 and 61% AMF recombined butter inoculated with 2 log CFU/g *C. guilliermondii* during 21 days of storage at 7 and 22 °C. Data points represent the average of three NMR measurements. Error bars denote standard deviation.

The D_{43} depicted in Figure 5.8 also follows the conclusions given in Paragraph 5.3.2. Refrigeration aided in keeping product stability, reflected in the similar D_{43} values measured during 21 days in both 82 and 61% AMF recombined butter. Storage at ambient temperature influenced emulsion destabilization, visible in the increase in D_{43} during time, especially in 61% AMF recombined butter.

In emulsions, microorganisms generally occupy the water phase (Brocklehurst and Wilson, 2000). Figure 5.9 shows a confocal microscopy image of 82% AMF recombined butter inoculated with 2 log CFU/g *C. guilliermondii* at day 0. It demonstrates that *C. guilliermondii* was present solely in the water phase of the W|O emulsion. Verrips and Zaalberg (1980), while assessing the intrinsic microbial stability of W|O emulsions, claimed that only a very small fraction (one of 10^6 or 10^7) of the water droplets with a radius larger than that of the microorganism actually contain a microorganism. Although the proliferation of *C. guilliermondii* wasn't followed up by confocal microscopy for the duration of the experiment, from Figure 5.9 it was observed that most droplets weren't inhabited by the yeast and of those who were, the droplets with most microorganisms were larger than 10 µm in diameter.

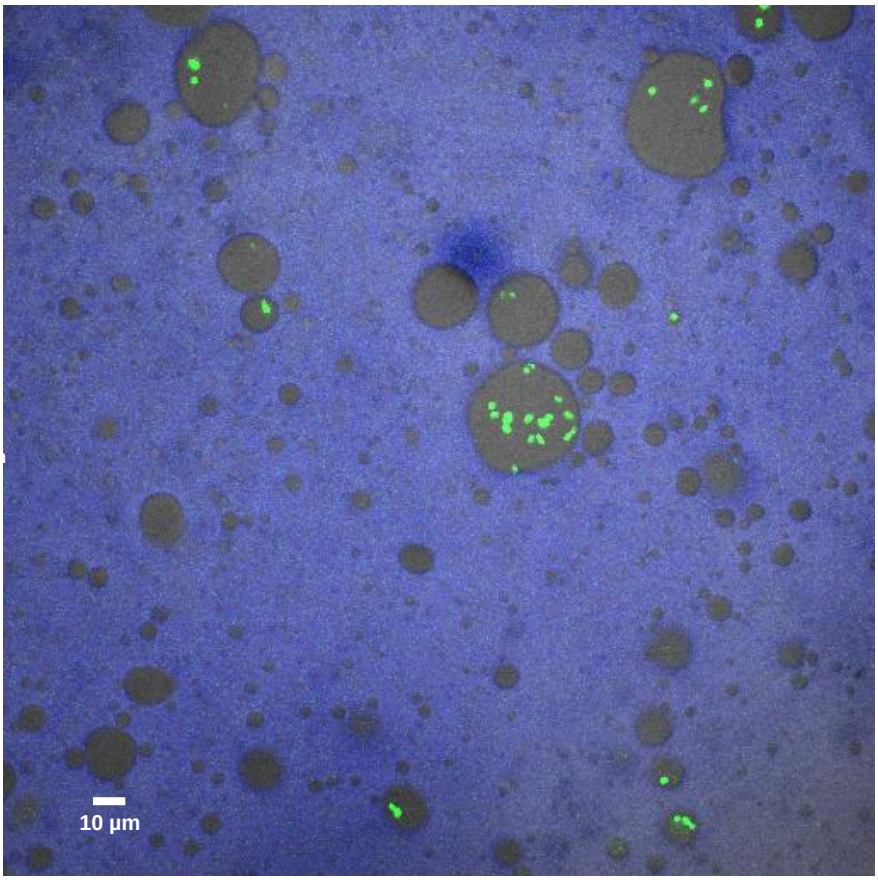


Figure 5.9. CLSM image showing GFP-tagged *C. guilliermondii* (green) in 82% AMF recombined butter stained with Nile Red (blue) on day 0 of storage period.

5.3.4. Growth of *C. guilliermondii* in 82 and 61% AMF recombined butter

Figure 5.10 shows the results of a challenge test with 2 log CFU/g of *C. guilliermondii* in 82 and 61% AMF recombined butter during 21 days at 7 and 22 °C. This trial period was chosen based on research done by Holliday et al. (2003) where the viability of *Salmonella, Escherichia coli* O157:H7 and *L. monocytogenes* in yellow fat spreads was monitored for 21 days as

affected by storage temperature. No growth of *C. guilliermondii* was observed in 82% AMF recombined butter at 7 °C during 21 days of storage. This was expected as the DSD in the samples remained similar during 21 days, and the number of vulnerable droplets was low (ca 5%) and didn't increase during time. In 82% AMF recombined butter at 22 °C, the maximum count of *C. guilliermondii* was observed on day 14, reaching 3.5 log CFU/g, followed by a decline in yeast count until the end of the trial period. DSD in these samples shifted slightly during 21 days (5 to 17% vulnerable droplets), thus allowing some space for proliferation. However, the CFU/g counts didn't increase to a large extent. This might have happened due to the depletion of nutrients in isolated water droplets, where the present nutrients might not have been sufficient to allow for further multiplication but only for maintenance of the cells (Verrips, 1989).

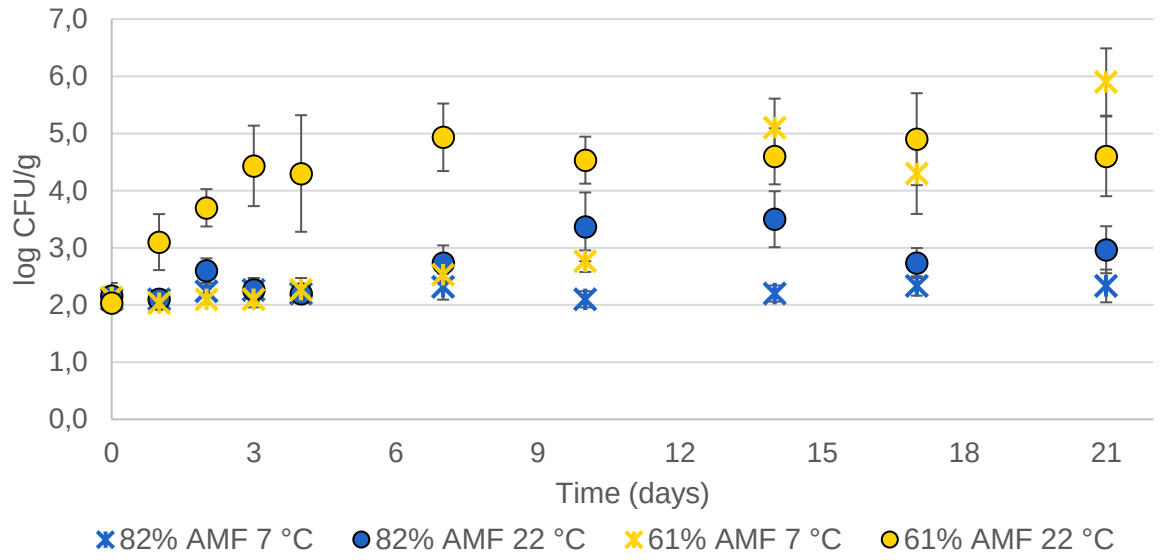


Figure 5.10. Growth of *C. guilliermondii* in 82 and 61% AMF recombined butter at 7 and 22 °C during 21 days. Data points represent the average of three replicates. Error bars denote standard deviation.

C. guilliermondii grew to maximum levels of 5.9 log CFU/g and 5.0 log CFU/g in 61% AMF recombined butter at 7 and 22 °C, respectively. Until day 10 of storage period, the CFU/g counts at 22 °C increased rapidly and then stabilized throughout the remainder of the storage period. Although the proportion of vulnerable droplets largely increased (18 to 35%) during 21 days, most likely it is the lack of nutrients that influenced the cessation of growth. The rapid growth of *C. guilliermondii* in 61% AMF recombined butter at 22 °C could have been a consequence of the destabilization of the emulsion structure and/or incubation at high temperature. At 7 °C, the proportion of vulnerable droplets was about 15% but remained similar throughout 21 days. Growth was much slower than at 22°C which can be explained by the lower temperature. Nevertheless, similar CFU/g counts than the ones observed at 22°C were observed in these samples at the last part of the storage period.

Fat can act as a barrier to microbial growth in fatty products like butter and margarine, so systems with a continuous lipid phase are usually more stabile than systems with a continuous water phase. The decrease in fat content in many products can have consequences on their microbiological stability, as often they consist of coarser water droplets. (van Zijl and Klapwijk, 2000). Our results indicate that a reduced fat content in recombined butter indeed negatively influenced microbial stability.

The physical structure of foods can have an effect on the local chemical environment perceived by microbial cells (Robins and Wilson, 1994). Physical restrictions on growth in reduced fat products are less severe than in full fat products. These types of products usually depend on preservative usage to keep microbial stability because a portion of the fat is replaced by water structuring agents such as starch (Varnam and Sutherland, 1994). De Mot et al. (1984) investigated the starch degradation and amylase production by non-ascomycetous yeast species and found that of 29 *Candida* strains, 18 strains were able to hydrolyze starch in media where it was the sole carbon source. In our study, 61% AMF recombined butter was made using modified starch as a structuring agent. The higher yeast counts in 61% AMF recombined butter compared to 82% AMF recombined butter might have occurred due to the presence of starch in the water phase, serving as an additional carbon source, in addition to the increased proportion of vulnerable water droplets allowing for yeast proliferation. It is also important to mention that several microorganisms can utilize fatty acids as a carbon source, like some lipolytic micrococci and the yeast *Yarrowia lipolytica*. In theory, they are able to grow out of the water droplet by metabolizing the fatty acids around it and have an unlimited amount of nutrients at their disposal. We performed a lipolysis test on Tributyrin agar and concluded that our *C. guilliermondii* strain wasn't able to metabolize fatty acids, thus the only available nutrients for the growth of this yeast strain were those present in the aqueous phase.

CONCLUSION

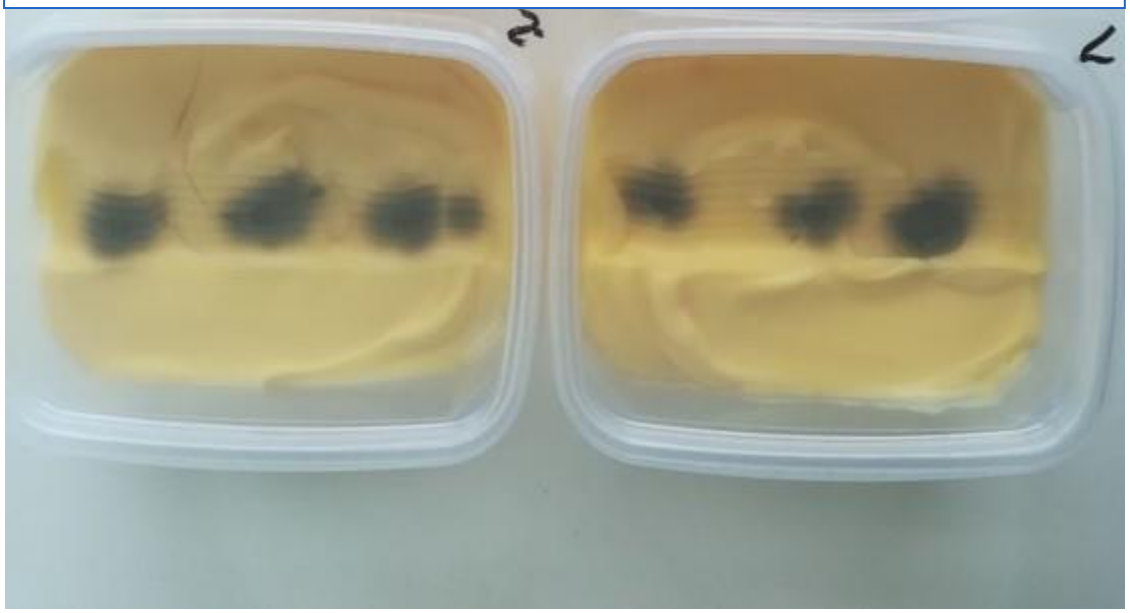
Foods based on oils and fats represent a large proportion of the energy intake in the diet of consumers in most of the world. In fat-continuous W | O emulsions, such as recombined butter, the water is present as well-dispersed fine droplets throughout the fat phase. The DSD, composition of the aqueous phase and keeping temperature influence the ability of microorganisms to grow in the isolated water droplets.

This study confirmed that the proportion of microbiologically vulnerable water droplets (diameter > 10 µm) increases as Ultra Turrax rotation speed during emulsification decreases. Also, stabile (diameter < 10 µm) water droplets were more likely to occur in 82% AMF recombined butter than in 61% AMF butter, irrespective of rotation speed during emulsification. Refrigeration for 21 days at 7 °C contributed to keeping the proportion of microbiologically vulnerable water droplets stabile, in both 82 and 61% AMF recombined butter. However, growth of *C. guilliermondii* was still observed in 61% AMF butter (containing a large proportion of vulnerable droplets from the start) at 7 °C, indicating that the proportion of vulnerable droplets at the time of production could also play a role in product stability. At 22 °C, the proportion of vulnerable droplets in both 82 and 61% AMF recombined butter increased during 21 days of storage. log CFU/g counts of *C. guilliermondii* increased at 22 °C, especially in 61% AMF recombined butter, which could have been a consequence of emulsion destabilization in combination with the elevated keeping temperature.

The results of this study could be of interest to researchers investigating microbial behavior in W│O emulsions and also to facilities in need of a laboratory scale emulsification method that enables microbiological challenge testing in W│O emulsions.

CHAPTER 6

Sensitivity of various emulsion spoiling molds towards sorbic acid and validation of sorbic acid distribution model in industrially produced W│O emulsions



SUMMARY

Yeasts and molds are among the most frequently encountered spoilage organisms associated with W│O emulsions. Spectrophotometric assay methods based on optical density measurements are increasingly utilized in mycological growth studies related to food matrices. The goal of the study was to elucidate sorbic acid MIC values for three emulsion spoiling molds in structured semi-solid media and validate those findings with the aid of the sorbic acid distribution model proposed in Chapter 2 in industrially produced W│O emulsions.

Semi-solid media of varying pH and concentrations of sorbic acid was inoculated with *Aspergillus niger, Cladosporium ramotenellum* and *Penicillium commune* in 96-multi well plates. Growth was followed at 7 °C for 30 days and at 22 °C for 7 days with VERSAmax™ Microplate Reader. The obtained MICs were then validated by inoculating 40 and 70% fat W│O emulsions with the above mentioned molds and growth was followed at 7 °C and 22 °C during 182 days. The inhibitory aqueous sorbic acid concentrations in W│O emulsions, $(HA)_{aq,eq}$ were calculated over the proposed sorbic acid distribution model taking into consideration the pH, mass fraction of lipid phase and solid fat content (SFC). Considering that sorbic acid preferentially partitions into the lipid phase of W│O emulsions, it is crucial to know exact $(HA)_{aq,eq}$ values during product formulation in order to prevent or retard product deterioration.

The results showed that the spectrophotometric measurement method was suitable for screening of mold sensitivity towards sorbic acid in semi-solid media. The MICs of total aqueous sorbic acid increased as pH of the YEG media increased. MICs of sorbic acid were lower in industrially produced W│O emulsions than in YEG media, confirming the importance of validation of microbiological preservative sensitivity assays in real food matrices.
The information obtained in this study is fundamentally valuable to emulsion producing facilities.

6.1. Introduction

Yeasts and molds are among the most frequently encountered spoilage organisms associated with W|O emulsions.The pH of W|O emulsions is rarely sufficiently low to solely be responsible for inhibiting fungal growth. Nevertheless, the pH value of the product is critical in product formulation, particularly when combined with weak organic acid preservatives, such as sorbic acid. Sorbic acid greatly reduces, but doesn't eliminate the risk of mold growth (Sofos and Busta, 1981).

Spectrophotometric assay methods based on optical density (OD) measurements are increasingly utilized in mycological growth studies related to food matrices (Schnurer, 1993; Rossi-Rodrigues et al., 2009; Aldars-García et al., 2018; Debonne et al., 2018). This technique has provided rapid growth results that are expressed in OD units (Medina et al., 2012).

The goal of this study was to elucidate sorbic acid MIC values for three emulsion spoiling molds in YEG semi-solid media at varying pH values and validate the obtained MIC values on industrially produced W|O emulsions with the aid of the sorbic acid distribution model in Chapter 2, taking into consideration the SFC of the emulsions.

The results from the industrial validation confirm the importance of calculating exact $(HA)_{aq,eq}$ during product development. The results also emphasize the importance of validation of microbiological preservative sensitivity studies results obtained in culture media on real food matrices.

6.2. Materials and methods

6.2.1. Cultivation of mold strains

Freeze dried cultures *Cladosporium ramotenellum* and *Pencillium commune* were obtained from The Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands) while *Aspergillus niger* (P1118) was obtained from the internal culture collection at UGent. The first two mentioned molds were isolated and identified from W|O emulsions made by the emulsion producing facility. The microorganisms were resuscitated, as per the supplier's instructions, by suspending the freeze-dried material in a sterile test tube with PPS, then by shaking gently and leaving at 22 °C for 6 hours. This suspension was poured on Malt Extract Agar (MEA, Oxoid, United Kingdom) plates and incubated at 22 °C for 1 week (Figure 6.1).

Figure 6.1. From left to right: *A. niger, Cl. ramotenellum and P. commune* on MEA. Incubation for 7 days at 22 °C.

6.2.2. Collecting mold spores

Fresh viable mold spores were obtained every 2 weeks by re-plating the spores mentioned in Paragraph 6.2.1. on MEA. Mold spores were collected by scraping the fully grown molds from MEA and suspending them in a series of solutions. The purpose of the procedure is to gather as many spores as from the MEA plate and to remove the hyphal fragments of the molds. Tween 80 solution consisted of 0.1 g of Tween 80 (Sigma Aldrich, Germany) dissolved in 100 mL of distilled water. Phosphate-Buffered Saline (PBS) was made by dissolving 1 pellet of solid PBS (Sigma Aldrich, USA) in 100 mL of distilled water. Afterwards, a 50:50 mixture of the above mentioned PBS and Tween 80 solutions was prepared.

First, 5 mL of Tween 80 solution was poured on the MEA plate and the fungal matter was scraped loose with a sterile loop. This was performed 3 times. The obtained spore suspension was filtered through a sterile cotton filter. The filtrate was then centrifuged for 15 minutes at 8000 rpm at 4 °C. After centrifugation, the supernatant was removed and the remaining spore pellet resuspended in 25 mL of the 50:50 PBS/Tween 80 solution and again centrifuged for 15 minutes at 8000 rpm at 4 °C. Then, the pellet was resuspended in PBS and centrifuged under the same conditions mentioned above. This step was repeated twice, but centrifugation was omitted the second time. The exact concentration of spores was determined with the aid of the Thoma counting chamber with 0.01 mm chamber depth (Marienfeld, Germany). The final spore count was in the order of magnitude of ca. 10^7 spores/mL for all the three mold strains.

6.2.3. Preparation of phosphate buffers for Yeast Extract Glucose (YEG) semi-solid media

Phosphate buffers at pH 3.5, 4.5, 5.5, which were used as solvents for YEG media used in the OD measurements, were prepared according to European Pharmacopoeia 7.0 (Council of Europe, 2004). For further details about phosphate buffer preparation, please refer to Chapter 2, Paragraph 2.2.3.1.

6.2.4. Media preparation for optical density (OD) measurements

YEG semi-solid media was prepared by dissolving 0.05 g/L $MgSO_4$ (Sigma Aldrich, USA), 1.2 g of bacteriological agar (Oxoid Ltd., UK), 20 g/L of yeast extract (Oxoid Ltd., UK) and 20 g/L of glucose (Sigma Aldrich, USA) in 1 L of phosphate buffers described in Paragraph 6.2.3. This non-selective fungal growth medium was adapted from the Yeast Extract Sucrose (YES) semi-solid media used by Medina et al. (2012).

Varying potassium sorbate quantities were weighed in separate sterile test tubes and dissolved in 10 mL of YEG pH 3.5, 4.5, 5.5, because it wasn't possible to add the desired quantities of potassium sorbate directly inside 96 multi-well plates. Potassium sorbate was used instead of sorbic acid due to its higher solubility in water (Sofos and Busta, 1981). The concentration range of sorbic acid in YEG pH 3.5 was 0 – 1000 mg/kg (0 – 1340 mg/kg potassium sorbate), while for YEG pH 4.5 and YEG pH 5.5 it was 0 – 2000 mg/kg (0 – 2680 mg/kg potassium sorbate). It was not possible to dissolve more than 1000 mg/kg sorbic acid in YEG pH 3.5 due to the appearance of white precipitate. Exact sorbic acid concentrations in preliminary and follow-up optical density growth experiments in 96 multi-well plates (Greiner CELLSTAR®; Sigma Aldrich, USA) are elaborated in Paragraph 6.2.5.

6.2.5. $((HA)_{aq,eq}+(A^-)_{aq,eq})$ in preliminary and follow-up OD experiments in YEG media

The 12 columns in 96 multi-well plates contained different sorbic acid concentrations, while the 8 rows were used as replicates. In the columns indicated in orange in Tables 6.1-6.5, YEG media prepared in sterile test tubes was placed, as described in Paragraph 6.2.4. These concentrations were then diluted once in the 96 multi-well plate (e.g. 1000 mg/L to 500 mg/L sorbic acid), indicated in white, in order to obtain several working sorbic acid concentrations. Common practice is to dilute one concentration several times directly in the 96 multi-well plate, but this approach was followed to improve result certainty.

Table 6.1. Total aqueous sorbic acid concentrations, ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) (mg/kg), in 96 multi-well plates in YEG pH 3.5. Orange cells represent YEG pH 3.5 media prepared in sterile test tubes while the adjacent white cells represent YEG pH 3.5 media prepared/diluted in the 96 multi-well plate. Eight replicates for every sorbic acid concentration.

	Columns											
	1	2	3	4	5	6	7	8	9	10	11	12
$(HA)_{aq,eq}+(A^-)_{aq,eq}$ (mg/kg)	0	0	1000	500	800	400	600	300	500	250	300	150

Table 6.2 Total aqueous sorbic acid concentrations, ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) (mg/kg), in 96 multi-well plates in YEG pH 4.5 and YEG pH 5.5. Orange cells represent YEG pH 4.5 and YEG pH 5.5 media prepared in sterile test tubes while the adjacent white cells represent YEG pH 4.5 and YEG pH 5.5 media prepared/diluted in the 96 multi-well plate. Eight replicates for every sorbic acid concentration.

	Columns											
	1	2	3	4	5	6	7	8	9	10	11	12
$(HA)_{aq,eq}+(A^-)_{aq,eq}$ (mg/kg)	0	0	2000	1000	1500	750	1200	600	1000	500	400	200

After assessing the results of the preliminary screening of MICs necessary to inhibit the growth of *A. niger*, *Cl. Ramotenellum* and *P. commune,* follow-up experiments were devised with a narrowed sorbic acid concentration range (Table 6.3-6.5).

Table 6.3. Total aqueous sorbic acid concentrations, ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) (mg/kg), in 96 multi-well plates in follow-up MIC experiments for *A. niger*. Orange cells represent YEG media prepared in sterile test tubes while the white cells represent YEG media prepared/diluted in the 96 multi-well plate. Eight replicates for every sorbic acid concentration.

$(HA)_{aq,eq}+(A^-)_{aq,eq}$ (mg/kg)	Columns											
	1	2	3	4	5	6	7	8	9	10	11	12
YEG pH 3.5	0	0	150	75	120	60	30	100	50	80	40	20
YEG pH 4.5	0	0	400	375	350	325	300	275	250	225	200	175
YEG pH 5.5	0	0	1200	1175	1150	1125	1100	1075	1050	1025	1000	/

Table 6.4. Total aqueous sorbic acid concentrations, ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) (mg/kg), in 96 multi-well plates in follow-up MIC experiments for *Cl. ramotenellum*. Orange cells represent YEG media prepared in sterile test tubes while the white cells represent YEG media prepared/diluted in the 96 multi-well plate. Eight replicates for every sorbic acid concentration.

$(HA)_{aq,eq}+(A^-)_{aq,eq}$ (mg/kg)	Columns											
	1	2	3	4	5	6	7	8	9	10	11	12
YEG pH 3.5	0	0	150	75	120	60	30	100	50	80	40	20
YEG pH 4.5	0	0	800	750	700	675	650	600	/	/	/	/
YEG pH 5.5	0	0	1200	1150	1100	1050	1000	900	800	/	/	/

Table 6.5. Total aqueous sorbic acid concentrations, ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) (mg/kg), in 96 multi-well plates in follow-up MIC experiments for *P. commune*. Orange cells represent YEG media prepared in sterile test tubes while the white cells represent YEG media prepared/diluted in the 96 multi-well plate. Eight replicates for every sorbic acid concentration.

$(HA)_{aq,eq}+(A^-)_{aq,eq}$ (mg/kg)	Columns											
	1	2	3	4	5	6	7	8	9	10	11	12
YEG pH 3.5	0	0	200	100	50	/	/	/	/	/	/	/
YEG pH 4.5	0	1000	500	250	375	350	325	300	275	225	400	200

6.2.6. OD measurements in YEG media

Each well in the 96 multi-well plate was filled with 200 µL YEG media inoculated with approximately 5×10^2 spores of *A. niger, Cl. Ramotenellum* or *P. commune*. One 96 multi-well plate contained YEG media of one pH value and was inoculated with one microorganism to avoid cross-contamination. 96 multi-well plates were prepared in duplicate and stored at 7 °C for 30 days and 22 °C for 7 days. VERSAmax^{TM} Microplate Reader (Molecular Devices, USA) was used for OD measurements . OD was measured at 595 nm wavelength and read every day during 30 days at 7 °C or three times per day during 7 days at 22 °C. During interpretation of the results, an increase of 0.1 OD from the initial measurement (T_0) was considered as growth. This corresponds to the tripling in size of a mold spore (Medina et al., 2012).

6.2.7. Validation in W | O emulsions

After obtaining the MIC values in YEG media, several batches of W | O emulsions were produced in order to validate the determined MICs. W | O emulsions with two fat percentages (40 and 70% fat), two pH levels (3.5 and 4.5) and three sorbic concentrations were produced. Sorbic acid concentrations that were applied were calculated as mg/kg inhibitory aqueous sorbic acid, $(HA)_{aq,eq}$. The applied $(HA)_{aq,eq}$ were (i) 0 mg/kg, (ii) 159-198 mg/kg or (iii) 258-333 mg/kg. Concentrations under (iii) correspond to the MIC values determined in OD experiments. These MICs were slightly different than the MICs in OD experiments because it was not possible to predict the pH and SFC of W | O emulsions before production. $(HA)_{aq,eq}$ is dependent on the SFC because the latter influences the partitioning process (Chapter 2). SFC measurements were performed at 5 °C and 20 °C because of the storage settings in the emulsion producing facility, although the experiments in this study were performed at 7 °C and 22 °C.

The produced W | O emulsions (± 100 g) were divided into plastic tubs and five tubs (replicates) per experimental condition were surface inoculated with *A.niger Cl. ramotenellum* or *P. commune* by pipetting three 20 µL drops, each containing 100 spores of either of the above mentioned mold strains. Prior to surface inoculation, the surface of the W | O emulsions was scraped off with a knife in order to mimic a common manner in which the consumer might contaminate the product. Figure 6.2 shows an example of a surface inoculated W | O emulsion. Inoculated W | O emulsions were stored at 7 °C and 22 °C and growth was followed for 182 days.

Table 6.6 Characteristics of W|O emulsions used for MIC validation in real food matrix. For explanation of the factors, refer to List of symbols. Recovery of sorbic acid by HPLC- 96-98%.

Code	r	pH_{meas}	f_{tot} 5 °C	f_{tot} 20 °C	$(HA)_{tot,eq}$ (mg/kg)	$(HA)_{tot,eq}$ (mg/kg)	$(HA)_{aq,eq}$ 5 °C (mg/kg)	$(HA)_{aq,eq}$ 20 °C (mg/kg)
		Measured	Measured	Measured	Added	Measured	Expected	Expected
42	0.4002	3.9	0.0993	0.0622	0	0	0	0
43	0.4002	5.0	0.0994	0.0563	0	0	0	0
44	0.4002	3.7	0.0980	0.0601	388	381	219	203
45	0.4002	4.8	0.1006	0.0600	440	425	182	171
46	0.4002	3.6	0.0989	0.0575	545	530	308	283
47	0.4002	4.8	0.0981	0.0569	851	821	350	330
48	0.7001	3.7	0.1780	0.0841	0	0	0	0
49	0.7001	4.4	0.1821	0.0805	0	0	0	0
50	0.7001	3.6	0.1839	0.0823	522	500	230	197
51	0.7001	4.4	0.1852	0.0823	552	530	232	200
52	0.7001	3.7	0.1815	0.0852	739	709	324	280
53	0.7001	4.4	0.1837	0.0851	970	955	418	362

Figure 6.2. Three spot inoculation (100 spores/20 µL) of W|O emulsions used for validation of MIC sorbic acid values obtained by OD measurements.

6.3. Results and discussion

6.3.1. OD measurements in YEG media

Tables 6.7 – 6.20 show the effect of sorbic acid on growth of *A. niger, Cl. ramotenellum* and *P. commune* in YEG pH 3.5, 4.5, 5.5 at 7 °C and 22 °C. The sensitivity of microorganisms towards preservatives is commonly tested in liquid culture media, but it was chosen to inoculate the fore mentioned molds in semi-solid media in order to add structure to the matrix. It was thought that this would be a closer representation of the structured matrix of food W|O emulsions than if only liquid media was used. The above mentioned tables are also visualized as growth curves in Annex 1 (Figure A.1-A.6).

6.3.1.1. Effect of sorbic acid on *A. niger* in YEG pH 3.5, 4.5, 5.5 at 7 and 22 °C

In YEG pH 3.5 at 7 °C during 30 days, *A. niger* was inhibited by the low pH and keeping temperature and didn't grow even at 0 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$). In YEG pH 4.5 and 5.5 at 7 °C, growth was observed at 0 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$), but the lowest tested concentration of 200 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) inhibited growth in both systems (Table 6.7). Table 6.8 shows the time of the onset of growth (days) of *A. niger* and the maximum OD reached at selected sorbic acid concentrations in YEG pH 3.5, 4.5, 5.5 at 7 °C during 30 days.

Table 6.7. Effect of sorbic acid on growth of *A. niger* in YEG pH 3.5, 4.5, 5.5 during 30 days at 7 °C. "G" signifies growth and "NG" signifies no growth at a known concentration. Minimum 16 replicates per condition.

pH	$((HA)_{aq,eq}+(A^-)_{aq,eq})$ (mg/kg)									
3.5	1000	800	600	500	400	300	250	150	0	
	NG	NG	NG	NG	NG	NG	NG	NG	NG	
4.5	2000	1500	1200	1000	750	600	500	400	200	0
	NG	NG	NG	NG	NG	NG	NG	NG	NG	G
5.5	2000	1500	1200	1000	750	600	500	400	200	0
	NG	NG	NG	NG	NG	NG	NG	NG	NG	G

Table 6.8. Growth onset time (days) of *A. niger* and maximum OD reached at selected sorbic acid concentrations in YEG pH 3.5, 4.5, 5.5 during 30 days at 7 °C. "∞" signifies onset of growth hadn't occurred during the trial period.

A.niger	pH	$((HA)_{aq,eq}+(A^-)_{aq,eq})$ (mg/kg)	Growth onset time (days)	Maximum OD ()
7 °C	3.5	0	∞	0.13
	3.5	150	∞	0.13
	4.5	0	12.7	0.27
	4.5	200	∞	0.13
	5.5	0	21.6	0.36
	5.5	200	∞	0.13

In preliminary experiments in YEG pH 3.5 at 22 °C, growth of *A. niger* was inhibited at 150 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) (Table 6.9). Thus, concentrations lower than the fore mentioned were chosen to be tested in the follow-up experiments, however growth was observed at all lower concentrations, including 150 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$).

Table 6.9. Effect of sorbic acid on growth of *A. niger* in YEG pH 3.5, 4.5, 5.5 during 7 days at 22 °C. "(1)" signifies results from preliminary and "(2)" signifies results from follow-up experiments. "G" signifies growth and "NG" signifies no growth at a known concentration. At least eight replicates per condition.

pH	$((HA)_{aq,eq}+(A^-)_{aq,eq})$ (mg/kg)									
3.5 (1)	1000	800	600	500	400	300	250	150	0	0
	NG	NG	NG	NG	NG	NG	NG	NG	G	G
3.5 (2)	150	120	100	80	75	60	50	40	30	20
	G	G	G	G	G	G	G	G	G	G
4.5 (1)	2000	1500	1200	1000	750	600	500	400	200	0
	NG	NG	NG	NG	NG	NG	NG	NG	G	G
4.5 (2)	400	375	350	325	300	275	250	225	200	175
	G	G	G	G	G	G	G	G	G	G
5.5 (1)	2000	1500	1200	1000	750	600	500	400	200	0
	NG	NG	G	G	G	G	G	G	G	G
5.5 (2)	1200	1175	1150	1125	1110	1075	1050	1025	1000	0
	G	G	G	G	G	G	G	G	G	G

In preliminary experiments in YEG pH 4.5 at 22 °C during 7 days, growth of *A. niger* was inhibited by 400 mg/kg $((HA)_{aq,eq}+(A^-)_{aq,eq})$, so concentrations between 400 mg/kg (NG) and 200 mg/kg (G) were tested in the follow-up experiments. However, growth was observed even at 400 mg/kg $((HA)_{aq,eq}+(A^-)_{aq,eq})$ in the latter experiments. In preliminary experiments in YEG pH 5.5 at 22 °C during 7 days, growth of *A. niger* was inhibited by 1500 mg/kg $((HA)_{aq,eq}+(A^-)_{aq,eq})$. In the follow-up experiments in YEG pH 5.5 at 22 °C, concentrations of 1200-1000 mg/kg $((HA)_{aq,eq}+(A^-)_{aq,eq})$ were erroneously chosen and *A. niger* growth occurred at all the tested concentrations. Table 6.10 shows the time of the onset of growth (days) of *A. niger* and the maximum OD reached at selected sorbic acid concentrations in YEG pH 3.5, 4.5, 5.5 at 22 °C during 7 days.

Table 6.10. Growth onset time (days) of *A. niger* and maximum OD reached at selected sorbic acid concentrations in YEG pH 3.5, 4.5, 5.5 during 7 days at 22 °C. "(1)" signifies results from preliminary and "(2)" signifies results from follow-up experiments."∞" signifies onset of growth hadn't occurred during the trial period.

A. Niger	pH	$((HA)_{aq,eq}+(A^-)_{aq,eq})$ (mg/kg)	Growth onset time (days)	Maximum OD (-)
22 °C (1)	3.5	0	2.6	2.14
	3.5	150	∞	0.13
	4.5	0	1.7	3.40
	4.5	200	3.6	3.18
	5.5	0	1.9	3.40
	5.5	1200	5.6	1.00
22 °C (2)	3.5	0	2.7	2.65
	3.5	150	6.8	0.63
	4.5	0	1.8	3.70
	4.5	400	4.9	2.32
	5.5	0	1.9	3.84
	5.5	1200	6.9	0.26

A.niger has been isolated and identified in butter since the middle of the past century (Sasaki, 1950). Pitt and Hocking (2009) stated that the minimum growth temperature of *A. niger* is 6-8 °C. Palacios-Cabrera et al. (2005) found that *A. niger*, *A. carbonarius* and *A. ochraceus* didn't grow in three growth media (Czapeck Yeast Extract Agar, Dichloran 18% Glycerol Agar and Malt extract 40% Glucose Agar) at 8 °C. No exact pH values of the media had been mentioned in the publication. In this study, low pH (3.5) and temperature (7 °C) aided to inhibit the growth of *A. niger* in YEG semi-solid media for 30 days.

Although the combination of low temperature and low pH was enough to inhibit *A. niger* growth in YEG pH 3.5 at 7 °C, at 22 °C sorbic acid needed to be added to prevent growth. Combining the observations from preliminary and follow-up experiments in YEG pH 3.5 at 22 °C, it was concluded that the MIC value for *A. niger* lies between 150 and 250 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$).

Heydaryinia et al. (2011) investigated the influence of sorbic acid on growth of *A. niger* in Yeast Extract Sucrose broth adjusted to pH 4.5 at ambient temperature for 40 days. They found that 1000 mg/kg of potassium sorbate, corresponding to 746 mg/kg sorbic acid, wasn't enough to completely inhibit growth, but postpone it for 23 days. In our study, growth in YEG pH 4.5 at 7 °C occurred only at 0 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$). In YEG pH 4.5 at 22 °C, combining the observations from preliminary and follow-up experiments, it was concluded that the MIC value for *A. niger* lies between 400 and 500 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$). The discrepancy between our findings and the findings of Heydaryinia et al. (2011) might lie in the difference between the type of strain used, different composition of growth media (15% sucrose in their experiments vs. 2% glucose in our experiments), duration of the experiment (40 days in their experiment vs. 7 days in ours) and the initial inoculum size (9-10 × 10^6 spores/mL in their experiment vs. 5 × 10^2 spores/well in ours).

In preliminary experiments in YEG pH 5.5 at 7 °C, no growth was observed at 200 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$). Combining the observations from preliminary and follow-up experiments in YEG pH 5.5 at 22 °C, it was concluded that the MIC value for *A. niger* lies between 1200 and 1500 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$).

Alcano et al. (2016) investigated the susceptibility of several *Aspergillus* spp. strains to acetic and sorbic acid in liquid culture media at pH 4.5, 5.0, 5.5, 6.5 for 6 days at 25 °C. They found that in culture media at pH 4.5, an 8 mM (897 mg/kg) concentration of sorbic acid was enough to inhibit the growth of 2 *A. niger* strains, while in culture media at pH 5.5 a concentration of 50 mM (5605 mg/kg) sorbic acid had the same effect. The inoculum level in the study was 10^3 spores per 4 mL tube of culture. Lukas (1964) reported that 0.1% potassium sorbate (746 ppm

sorbic acid) hindered germination of conidia of *A. niger* and 0.15% (1119 ppm sorbic acid) inhibited mycelial growth. Liewen and Marth (1984) also investigated the inhibition of several strains of *Penicillium* spp. and *Aspergillus* spp. strains by potassium sorbate. They found that when 10^7 *A. niger* spores were inoculated on YM agar at pH 5.5 containing various concentrations of potassium sorbate, 500 ppm of potassium sorbate was enough to inhibit *A. niger* growth at 25 °C. No growth was observed at 4 °C, even on agar without potassium sorbate. This is in accordance to our observation about the sensitivity of *A. niger* towards low temperature.

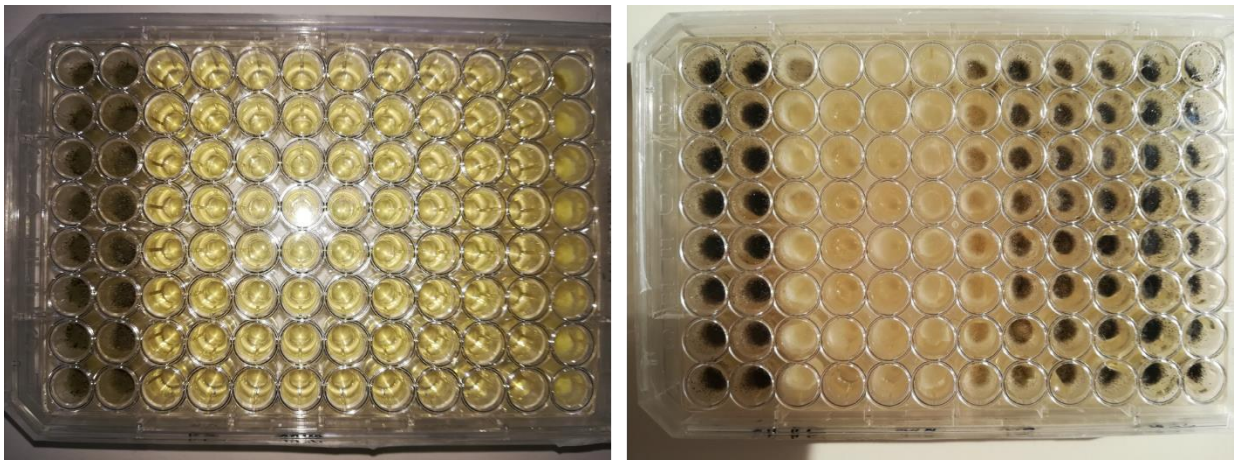


Figure 6.3. The effect of sorbic acid on growth of *A. niger in* preliminary experiments in YEG pH 4.5 (left) and follow-up experiments in YEG pH 4.5 (right) at 22 °C in 96-multi well plates. Varying concentrations of sorbic acid in columns, replicates in rows. For sorbic acid concentrations refer to Table 6.2 and 6.3.

6.3.1.2. Effect of sorbic acid on *Cl. ramotenellum* in YEG pH 3.5, 4.5, 5.5 at 7 and 22 °C

In YEG pH 3.5 at 7 °C, growth of *Cl. ramotenellum* was inhibited by 150 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) during 30 days (Table 6.11). Results from YEG pH 4.5 and YEG pH 5.5 have been split into two replicates (individual 96 multi-well plates) because the results differed between the two. In YEG pH 4.5 it was decided that the MIC will lie in the range of 600-750 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) although growth of *C. ramotenellum* was inhibited by lower concentrations in one of the replicates. The MIC in YEG pH 5.5 at 7 °C was also in the range between 600-750 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) although the same phenomenon was observed as described in YEG pH 4.5.

Table 6.11. Effect of sorbic acid on growth of *Cl.ramotenellum* in YEG pH 3.5, 4.5, 5.5 during 30 days at 7 °C. "R1" and "R2" signify replicate 1 and replicate 2, respectively. "G" signifies growth and "NG" signifies no growth at a known concentration. At least eight replicates per condition.

pH	$((HA)_{aq,eq}+(A^-)_{aq,eq})$ (mg/kg)									
3.5	1000	800	600	500	400	300	250	150	0	
	NG	NG	NG	NG	NG	NG	NG	NG	G	
4.5	2000	1500	1200	1000	750	600	500	400	200	0
(R1)	NG	NG	NG	NG	NG	NG	NG	NG	G	G
4.5	2000	1500	1200	1000	750	600	500	400	200	0
(R2)	NG	NG	NG	NG	NG	G	G	G	G	G
5.5	2000	1500	1200	1000	750	600	500	400	200	0
(R1)	NG	NG	NG	NG	NG	G	G	G	G	G
5.5	2000	1500	1200	1000	750	600	500	400	200	0
(R2)	NG	NG	NG	NG	NG	NG	G	G	G	G

Table 6.12 shows the time of the onset of growth (days) of *Cl. ramotenellum* and the maximum OD reached at selected sorbic acid concentrations in YEG pH 3.5, 4.5, 5.5 during 30 days at 7 °C.

Table 6.12. Growth onset time (days) of *Cl. ramotenellum* and maximum OD reached at selected sorbic acid concentrations in YEG pH 3.5, 4.5, 5.5 during 30 days at 7 °C. "R1" and "R2" signify replicate 1 and replicate 2, respectively. "∞" signifies onset of growth hadn't occurred during the trial period.

Cl. ramotenellum	pH	$((HA)_{aq,eq}+(A^-)_{aq,eq})$ (mg/kg)	Growth onset time (days)	Maximum OD (-)
7 °C	3.5	0	6.1	0.93
	3.5	150	∞	0.20
	4.5 (R1)	0	4.9	3.52
	4.5 (R1)	200	7.1	3.14
	4.5 (R2)	0	4.9	3.63
	4.5 (R2)	600	20.8	1.40
	5.5 (R1)	0	4.9	3.36
	5.5 (R1)	600	13.9	2.24
	5.5 (R2)	0	4.9	3.40
	5.5 (R2)	500	27.2	0.57

Combining the observations from preliminary and follow-up experiments in YEG pH 3.5, 4.5 and 5.5 at 22 °C, it was concluded that the MIC value for *Cl. ramotenellum* lies between 40-50, 500-600 and 750-800 mg/kg $((HA)_{aq,eq}+(A^-)_{aq,eq})$, respectively (Table 6.13). Table 6.14 shows the time of the onset of growth (days) of *Cl. ramotenellum* and the maximum OD reached at selected sorbic acid concentrations in YEG pH 3.5, 4.5, 5.5 during 7 days at 22 °C.

Table 6.13. Effect of sorbic acid on growth of *Cl. ramotenellum* in YEG pH 3.5, 4.5, 5.5 during 7 days at 22 °C. "(1)" signifies results from preliminary and "(2)" signifies results from follow-up experiments. "G" signifies growth and "NG" signifies no growth at a known concentration. "/" signifies an empty column in the 96-well plate. At least eight replicates per condition.

pH	$((HA)_{aq,eq}+(A^-)_{aq,eq})$ (mg/kg)									
3.5 (1)	1000	800	600	500	400	300	250	150	0	0
	NG	NG	NG	NG	NG	NG	NG	NG	G	G
3.5 (2)	150	120	100	80	75	60	50	40	30	20
	NG	NG	NG	NG	NG	NG	NG	G	G	G
4.5 (1)	2000	1500	1200	1000	750	600	500	400	200	0
	NG	NG	NG	NG	G	G	G	G	G	G
4.5 (2)	800	750	700	675	650	600				
	NG	NG	NG	NG	NG	NG				
5.5 (1)	2000	1500	1200	1000	750	600	500	400	200	0
	NG	NG	G	G	G	G	G	G	G	G
5.5 (2)	1200	1150	1100	1050	1000	900	800			
	NG	NG	NG	NG	NG	NG	NG			

Table 6.14. Growth onset time (days) of *Cl. ramotenellum* and maximum OD at selected sorbic acid concentrations in YEG pH 3.5, 4.5, 5.5 during 7 days at 22 °C. "(1)" signifies results from preliminary and "(2)" signifies results from follow-up experiments."∞" signifies onset of growth hadn't occurred during the trial period.

Cl. ramotenellum	pH	$((HA)_{aq,eq}+(A^-)_{aq,eq})$ (mg/kg)	Growth onset time (days)	Maximum OD (-)
22 °C (1)	3.5	0	3.8	2.39
	3.5	150	∞	0.13
	4.5	0	1.9	2.69
	4.5	1000	4.8	0.39
	5.5	0	2.2	2.79
	5.5	1200	6.2	0.37
22 °C (2)	3.5	0	2.7	2.49
	3.5	40	6.7	0.41
	4.5	0	1.7	2.60
	4.5	600	∞	0.13
	5.5	0	1.3	3.44
	5.5	800	∞	0.13

Strains of *Cladosporium* spp. are ubiquitous in distribution and commonly encountered on all kinds of plant, fungal and other debris. They are frequently isolated from soil, food, paint, textiles and other organic matter (Ellis 1971, 1976). Spores of *Cladosporium* spp. also represent the most common fungal components isolated from air (Farr et al., 1989; Flannigan 2001; Mullins 2001). Information about *Cl. ramotenellum* in literature is scarce. The microorganism was first known when Schubert et al. (2007) isolated one strain from hypersaline water and an additional strain from an air conditioning system. In the meantime, Bensch et al. (2015) identified *Cl. ramotenellum* in cheese, margarine, garfish, saline water and several plant species.

Pitt and Hocking (2009) stated that all common species of genus *Cladosporium* grow at temperatures near 0 °C. This explains why *Cladosporium* spp. have been found on chilled commodities. Bensch et al. (2012) found that the minimum temperature for growth of *Cl. donicanum,* Cl. fusiforme, *Cl. halotolerans* and *Cl. sphaerospermum* is between 4 °C and 10 °C. They also found that optimum and maximum growth temperatures for the above mentioned molds were 25 °C and 30 °C. In our study it was also observed that temperature had an effect on the growth of *Cl. ramotenellum* in YEG media. Namely, the onset of growth of the above mentioned mold at 0 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) occurred earlier at 22 °C than at 7 °C, irrespective of the YEG system in question (Table 6.12 and 6.14).

Also, an effect of pH on the growth of *Cl. ramotenellum* could be observed. Namely, the onset of growth was delayed when comparing growth at 0 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) in YEG pH 3.5 at 7 and 22 °C with the growth in YEG pH 4.5 and YEG pH 5.5. Mehra and Jaitly (1995) investigated the effect of pH on the growth and sporulation of some common fungi in city waste. They concluded that the dry weight in mg of *Cl. herbarum* on Czapek-Dox medium incubated at 29 °C during 7 days increased from 190 mg at pH 3.5, to 330 mg on pH 4.5, to 480 mg on pH 5.5. The increase of fungal matter with the increase in pH is in line with the findings in our study. It was observed that maximum OD values in YEG media with 0 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) increased as pH increased (Table 6.14).

Adding sorbic acid in YEG media either inhibited or retarded the growth of *Cl. ramotenellum*, at both 7 and 22 °C. Skirdal and Eklund (1993) investigated the effect of sorbic acid on *Cl. cladosporioides* in BHI at pH 4.1-7.6 in flat-bottomed 96-well tissue plates at 25 °C for 5 days and found that 0.3-18 mmol/L (33.6-2018.3 mg/L) was enough to inhibit the growth of the mold. More specifically, in the pH range of 4.1-5.5, which encompasses a part of the pH range from this study, less than 2.5 mmol/L (280.33 mg/L) of sorbic acid was enough to inhibit the growth of the mold above. Pitt and Hocking (2009) found that the minimum inhibitory concentration of sorbic acid for *Cl. cladosporioides* was 160 mg/L at 25 °C and pH 5.

Comparison of the MIC values obtained for *Cl. ramotenellum* in YEG media with the values in literature is a difficult task due to the lack of information in literature about this exact strain. However, we observed that as pH increased, so did the sorbic acid concentration necessary to inhibit *Cl. ramotenellum* increase.

6.3.1.3. The effect of sorbic acid on *P. commune* in YEG media pH 3.5, 4.5, 5.5 at 7 and 22 °C

In YEG pH 3.5 at 7 °C, 150 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) was sufficient to inhibit the growth of *P. commune.* 400 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) inhibited *P. commune* in YEG pH 4.5, while in YEG pH 5.5, growth was observed even at the highest applied concentration, 2000 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) (Table 6.15). Table 6.16 shows the time of the onset of growth (days) of *P. commune* and the maximum OD reached at selected sorbic acid concentrations in YEG pH 3.5, 4.5, 5.5 at 7 °C during 30 days of trial period.

Table 6.15. Effect of sorbic acid on growth of *P. commune* in YEG pH 3.5, 4.5, 5.5 during 30 days at 7 °C. "G" signifies growth and "NG" signifies no growth at a known concentration. 16 replicates per condition.

pH	$((HA)_{aq,eq}+(A^-)_{aq,eq})$ (mg/kg)									
3.5	1000	800	600	500	400	300	250	150	0	
	NG	NG	NG	NG	NG	NG	NG	NG	G	
4.5	2000	1500	1200	1000	750	600	500	400	200	0
	NG	NG	NG	NG	NG	NG	NG	NG	G	G
5.5	2000	1500	1200	1000	750	600	500	400	200	0
	G	G	G	G	G	G	G	G	G	G

Table 6.16. Growth onset time (days) of *P. commune* and maximum OD reached at selected sorbic acid concentrations in YEG pH 3.5, 4.5, 5.5 at 7 °C during 30 days. "∞" signifies onset of growth hadn't occurred during the trial period.

P. commune	pH	$((HA)_{aq,eq}+(A^-)_{aq,eq})$ (mg/kg)	Growth onset time (days)	Maximum OD (-)
7 °C	3.5	0	5.1	3.30
	3.5	150	∞	0.13
	4.5	0	5.1	3.34
	4.5	200	7.1	3.20
	5.5	0	4.8	3.19
	5.5	2000	19.1	1.57

Combining the observations from preliminary and follow-up experiments in YEG pH 3.5 and 4.5 at 22 °C, it was concluded that the MIC value for *P. commune* lies between 150 and 200 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) and 375-400 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$), respectively (Table 6.17). As observed at 7 °C, in YEG pH 5.5, growth occurred even at the highest applied concentration, 2000 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$). Table 6.18 shows the time of the onset of growth (days) of *P. commune* and the maximum OD reached at selected sorbic acid concentrations in YEG pH 3.5, 4.5, 5.5 during 7 days at 22 °C.

Table 6.17. Effect of sorbic acid on growth of *P. commune* in YEG pH 3.5, 4.5, 5.5 during 7 days at 22 °C. "(1)" signifies results from preliminary and "(2)" signifies results from follow-up experiments. "G" signifies growth and "NG" signifies no growth at a known concentration. At least eight replicates per condition.

pH	$((HA)_{aq,eq}+(A^-)_{aq,eq})$ (mg/kg)									
3.5 (1)	1000	800	600	500	400	300	250	150	0	0
	NG	NG	NG	NG	NG	NG	NG	G	G	G
3.5 (2)	200	100	50	0						
	NG	G	G	G						
4.5 (1)	2000	1500	1200	1000	750	600	500	400	200	0
	NG	NG	NG	NG	NG	NG	NG	NG	G	G
4.5 (2)	500	400	375	350	325	300	275	250	225	200
	NG	NG	G	G	G	G	G	G	G	G
5.5 (1)	2000	1500	1200	1000	750	600	500	400	200	0
	G	G	G	G	G	G	G	G	G	G

Penicillium spp. have been isolated from hard and semi-hard cheeses, butter, yoghurt, milk (Garnier et al., 2017). *Penicillium* species grow over a wide pH range, pH 3 to pH 8 (Onions and Brady, 1987) and some even at pH 2 and pH 3 like *P. citrinum*, *P citreonigrum*, *P. islandicum* (Wheeler et al., 1991). Pitt and Hocking (2009) stated that *P. commune* has an optimum for growth near 25 °C and a maximum for growth near 35 °C. In this study, *P. commune* grew faster at 22 °C than at 7 °C, irrespective of the pH used.

Table 6.18. Growth onset time (days) of *P. commune* and maximum OD reached at selected sorbic acid concentrations in YEG pH 3.5, 4.5, 5.5 during 7 days at 22 °C. "1" signifies results from preliminary and "2" signifies results from follow-up experiments.

P. commune	pH	$((HA)_{aq,eq}+(A^-)_{aq,eq})$ (mg/kg)	Growth onset time (days)	Maximum OD (-)
22 °C (1)	3.5	0	1.9	2.95
	3.5	150	6.0	1.18
	4.5	0	1.2	3.64
	4.5	200	2.5	3.57
	5.5	0	1.2	3.54
	5.5	2000	4.1	1.87
22 °C (2)	3.5	0	3.6	3.30
	3.5	100	4.8	1.67
	4.5	0	2.2	3.53
	4.5	375	2.9	3.29

Rehm (1961) stated that 200 – 1000 mg/kg sorbic acid was enough to inhibit the growth of *Penicillium* species at pH 3.5-5.7. In line with this observation, *P. commune* was also inhibited by 200 mg/kg $((HA)_{aq,eq}+(A^-)_{aq,eq})$ in YEG pH 3.5 at 22 °C, while at 7 °C the inhibitory concentration of sorbic acid was 150 mg/kg $((HA)_{aq,eq}+(A^-)_{aq,eq})$. It was also observed that, generally, the onset of growth was delayed and the final ODs were slightly lower at growth-permitting concentrations of $((HA)_{aq,eq}+(A^-)_{aq,eq})$, compared to the results at 0 mg/kg $((HA)_{aq,eq}+(A^-)_{aq,eq})$.

Marth et al. (1966) found that *P. roqueforti*, *P. notatum*, *P. frequentans* and *P. cyaneo-fulvum* grown at room temperature on Potato Dextrose Agar at pH 5.5, were able to grow in the presence of at least 1800 mg/kg of potassium sorbate (1343 mg/kg of sorbic acid). Liewen and Marth (1984) tested concentrations of potassium sorbate that permitted growth of various *Penicillium* strains on Yeast Malt Agar at pH 5.5 and 25 °C and found that the ability to grow in the presence of 3000 mg/kg of potassium sorbate (2239 mg/kg sorbic acid) is widespread among several strains of molds in the genus *Penicillium*. In this study, 2000 mg/kg ($(HA)_{aq,eq}+(A^-)_{aq,eq}$), the maximum permitted sorbic acid concentration in fat products (EC EU 1333/2008), couldn't inhibit the growth of *P. commune* in YEG pH 5.5 at 7 °C and 22 °C. Further experiments with higher concentrations of sorbic acid weren't performed because of the legal limitations mentioned above. However, it was again concluded that, the higher the pH of YEG media, the higher the sorbic acid concentration necessary to inhibit growth of *P. commune.*

6.3.2. Summary of sorbic acid MICs on *A. niger, Cl. ramotenellum* and *P. commune* in YEG media

Table 6.19 shows the summary of MICs of sorbic acid on *A. niger, Cl. ramotenellum* and *P. commune* observed in experiments discussed in Paragraphs 6.3.1.1. to 6.3.1.3. Organic acids such as sorbic acid dissociate in aqueous solutions and release hydrogen ions. This dissociation is key to prediction of the concentration of the undissociated form of the acid, which exhibits the predominant antimicrobial effect in foods (Eklund, 1983; van Zijl and Klapwijk, 2000). Effectiveness of the preservative is dependent on pH of the product, as the antimicrobial effect of the undissociated acid is much stronger than the dissociated acid (Suhr and Nielsen, 2004). This was the reason why it was important to express the ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) MICs in Table 6.19 as active aqueous sorbic acid, $(HA)_{aq,eq}$, in Table 6.20. The concentrations of $(HA)_{aq,eq}$ were calculated over Equation 1.9 (Henderson-Hasselbalch equation).

Table 6.19. Summary of MICs (mg/kg) of ($(HA)_{aq,eq}+(A^-)_{aq,eq}$) for *A. niger* (AN), *Cl. ramotenellum* (CR) and *P. commune* (PC) in YEG media at 7 °C and 22 °C.

($(HA)_{aq,eq}+(A^-)_{aq,eq}$) (mg/kg)	**AN**	**AN**	**CR**	**CR**	**PC**	**PC**
Temperature (°C)	7	22	7	22	7	22
YEG pH 3.5	0	150–250	< 150	40-50	150-250	150-200
YEG pH 4.5	0	400–500	600–750	< 600	200-400	375-400
YEG pH 5.5	0	1200-1500	600–750	750-800	> 2000	>2000

Table 6.20. Summary of MICs (mg/kg) of active sorbic acid, $(HA)_{aq,eq}$, for *A. niger* (AN), *Cl. ramotenellum* (CR) and *P. commune* (PC) in YEG media at 7 °C and 22 °C

$(HA)_{aq,eq}$ (mg/kg)	AN	AN	CR	CR	PC	PC
Temperature (°C)	7	22	7	22	7	22
YEG pH 3.5	0	142–237	< 142	37.9-47	142-237	142-189
YEG pH 4.5	0	256–320	384–480	< 384	128-256	240-256
YEG pH 5.5	0	181-226	90.6–113	113-120.8	> 302	>302

Sorbate is more effective in foods with low rather than high pH values (Bandelin, 1958; Bell et al., 1959; Lück, 1980; Sofos and Busta, 1981). When observing the results in Table 6.19, a pattern of increased sorbic acid efficiency at low pH values, for all three molds tested, on both temperatures, was noticed. However, when the observed MIC's were converted into $(HA)_{aq,eq}$ (Table 6.20), a clear pattern could only be observed in systems inoculated with *P. commune*.

Alcano et al. (2016) investigated the susceptibility of *Aspergillus* spp. to acetic and sorbic acid based on pH. They also concluded that there was an increase in the amount of acid, both for acetic acid and sorbic acid, necessary to inhibit the growth of the isolates with increasing pH values. However, their results showed that, when considering the amount of undissociated acid, the inhibitory concentration remained approximately constant. They explained that this happened because as the pH increased, there was a decrease in the amount of undissociated acid molecules and observed that for each increase of 0.5 in pH value, the acid concentrations needed to inhibit the same fungal isolate doubled. Our findings regarding the increasing amount of total sorbic acid necessary to inhibit molds as pH increases correspond to Alcano et al. (2016), but the inhibitory $(HA)_{aq,eq}$ in our experiments didn't follow a clear trend.

6.3.3. Validation of sorbic acid MICs on industrially produced W|O emulsions

The obtained $((HA)_{aq,eq}+(A^-)_{aq,eq})$ MICs (Table 6.19 and 6.20) were validated on 40 and 70% fat W|O emulsions at pH 3.5 and pH 4.5, produced in a pilot emulsion making plant in Vandemoortele NV, Belgium. In agreement with the emulsion producing facility, emulsions at pH 5.5 were not prepared due to the material-intensive experimental setup.

Three $(HA)_{aq,eq}$ were applied, (i) 0 mg/kg - to observe mold growth in unpreserved W|O emulsions, (ii) 171-232 mg/kg – an intermediate sorbic acid concentration and (iii) 283-418 mg/kg – $(HA)_{aq,eq}$ corresponding to the range of MICs obtained from YEG media. The $((HA)_{aq,eq}+(A^-)_{aq,eq})$ MIC's at pH 3.5 and 4.5 elucidated in the YEG media and the $((HA)_{aq,eq}+(A^-)_{aq,eq})$ applied in case (iii) above slightly differed because it was not possible to predict the SFC and final pH of the W|O emulsions before production.

The $(HA)_{tot,eq}$ to be added in the system to achieve the $(HA)_{aq,eq}$ mentioned under (ii) and (iii) were calculated over Equation 2.11. (See Chapter 2). Should the presence of a lipid phase in W│O emulsions not be taken into account while calculating the necessary $(HA)_{tot,eq}$ to be added to the product for the MIC results in YEG media to be validated (Chapter 6.3.2.), the $(HA)_{tot,eq}$ would be the same as the $((HA)_{aq,eq}+(A^-)_{aq,eq})$ MICs in YEG media. However, due to the preferential partitioning of sorbic acid into lipid phases, $(HA)_{tot,eq}$ needed to be higher than what was needed in the aqueous phase. This is governed by the K_p, the partition coefficient. Also, as postulated in Chapter 2, it was assumed that solid fat was inert, and this reasoning was applied in $(HA)_{aq,eq}$ calculations.

Both applied $(HA)_{aq,eq}$ levels completely inhibited the growth of *A. niger, Cl. ramotenellum* and *P. commune* in 40 and 70% fat W│O emulsions at all pH values at 7 and 22 °C during 182 days. The pH values shifted from the initial pH 3.5 and 4.5 of the water phase because of the interaction between the matrix phases and the solutes present in the emulsions. The lowest applied $(HA)_{aq,eq}$ that inhibited mold growth during 182 days was 171 mg/kg $(HA)_{aq,eq}$ in 40% fat W│O emulsions at pH 4.8 and 22 °C (Table 6.21). Because the effectiveness of weak organic preservatives increases with decreasing pH and microorganisms generally grow better at higher than at lower pH values and at room than at refrigeration temperature, it might be possible that even lower $(HA)_{aq,eq}$ than those tested could have inhibited growth of the tested molds in W│O emulsions.

The $(HA)_{aq,eq}$ MICs in W│O emulsions were lower than those elucidated in YEG media. This finding confirms the importance of validating preservative effectiveness results from laboratory culture media in real food matrices. In other words, the MIC results from YEG media proved to be “fail-safe”, i.e. if they were applied in W│O emulsions, the chance of product deterioration by the tested molds would be negligible.

Table 6.21. The effect of sorbic acid on *A. niger, Cl. ramotenellum* and *P. commune* in 40 and 70% W│O emulsions during 182 days at 7 °C and 22 °C. "G" signifies growth and "NG" signifies no growth.

7 °C						
Code	**r**	**Final pH**	**$(HA)_{aq,eq}$ (mg/kg)**	**A.niger**	***Cl. ramotenellum***	***P. commune***
42	0.4002	3.9	0	NG	G	G
43	0.4002	5.0	0	NG	G	G
44	0.4002	3.7	219	NG	NG	NG
45	0.4002	4.8	182	NG	NG	NG
46	0.4002	3.6	308	NG	NG	NG
47	0.4002	4.8	350	NG	NG	NG
48	0.7001	3.7	0	NG	NG	NG
49	0.7001	4.4	0	NG	G	NG
50	0.7001	3.6	230	NG	NG	NG
51	0.7001	4.4	232	NG	NG	NG
52	0.7001	3.7	324	NG	NG	NG
53	0.7001	4.4	418	NG	NG	NG
22 °C						
Code	**r**	**Final pH**	**$(HA)_{aq,eq}$ (mg/kg**	**A.niger**	***Cl. ramotenellum***	***P. commune***
42	0.4002	3.9	0	G	G	G
43	0.4002	5.0	0	G	G	G
44	0.4002	3.7	203	NG	NG	NG
45	0.4002	4.8	171	NG	NG	NG
46	0.4002	3.6	238	NG	NG	NG
47	0.4002	4.8	330	NG	NG	NG
48	0.7001	3.7	0	NG	G	NG
49	0.7001	4.4	0	G	G	NG
50	0.7001	3.6	197	NG	NG	NG
51	0.7001	4.4	200	NG	NG	NG
52	0.7001	3.7	280	NG	NG	NG
53	0.7001	4.4	362	NG	NG	NG

6.3.3.1. Growth of *A. niger* in 40 and 70% fat W│O emulsions at pH 3.5 and 4.5

A. niger didn't grow in unpreserved 40 and 70% fat W│O emulsions at 7°C (Table 6.21). This again emphasizes the sensitivity of *A. niger* towards low temperature (Palacios-Cabrera et al., 2005). In unpreserved W│O emulsions at 22 °C, *A. niger* grew faster in 40% than in 70% fat W│O emulsions (Figure 6.4). The coarser structure, increased space and more nutrients enabled the mold to grow more quickly in the low-fat product. As for the preserved W│O emulsions, the $(HA)_{aq,eq}$ MICs in W│O emulsions were lower than the $(HA)_{aq,eq}$ MICs elucidated in YEG media, at both pH's.

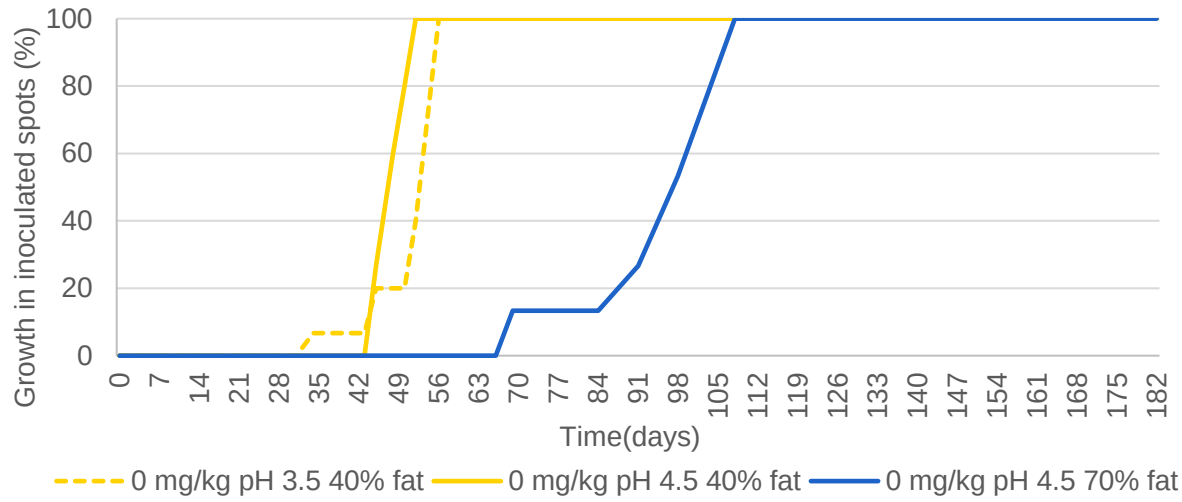


Figure 6.4. Growth in inoculated spots of *A. niger* in 40 and 70% fat W|O emulsions at pH 3.5 and 4.5 during 182 days at 22 °C.

6.3.3.2. Growth of *Cl. ramotenellum* in 40 and 70% fat W|O emulsions at pH 3.5 and 4.5

Cl. ramotenellum grew faster in 40% than in 70% fat W|O emulsions at both pH's at 7 °C (Figure 6.5), showing again that the presence of a lipid phase in W|O emulsions has an influence on mold growth. No growth was observed after 182 days in 70% fat W|O emulsions at pH 3.5, indicating that high fat content and low pH might help to inhibit the growth of *Cl. ramotenellum* in W|O emulsions.

Cl. ramotenellum was the only mold able to grow in both 40 and 70% fat W|O emulsions at both pH values at 22 °C (Figure 6.7). It grew faster in 40% fat W|O emulsions than in 70% fat W|O emulsions and at pH 4.5 than at pH 3.5. Figure 6.6 shows five replicates of 40% fat W|O emulsions at pH 3.5 and 4.5 inoculated with *Cl. ramotenellum* and incubated at 22 °C.

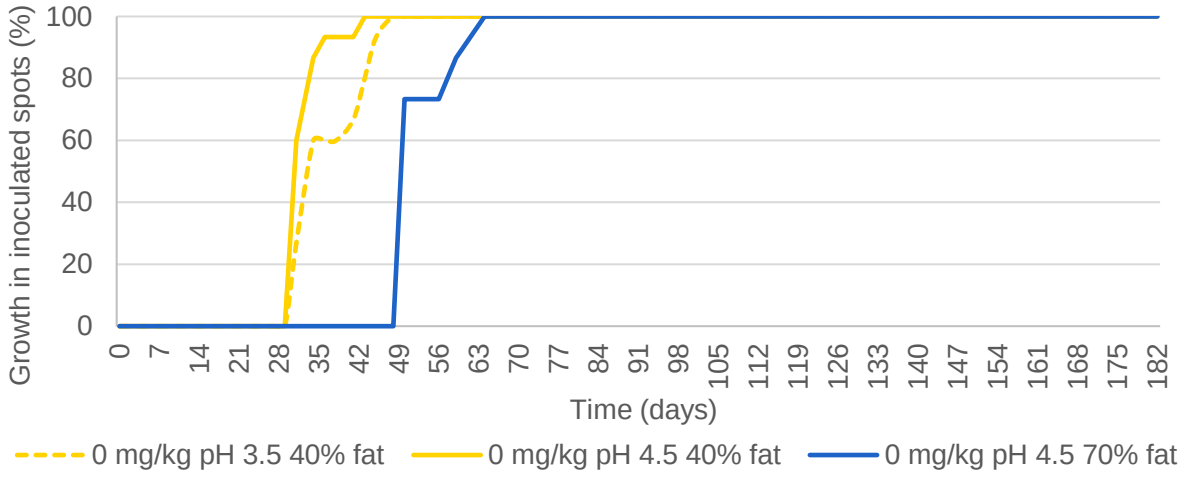


Figure 6.5. Growth (% inoculated spots) of *Cl. ramotenellum* in 40 and 70% W|O emulsions at pH 3.5 and 4.5 during 182 days at 7 °C.

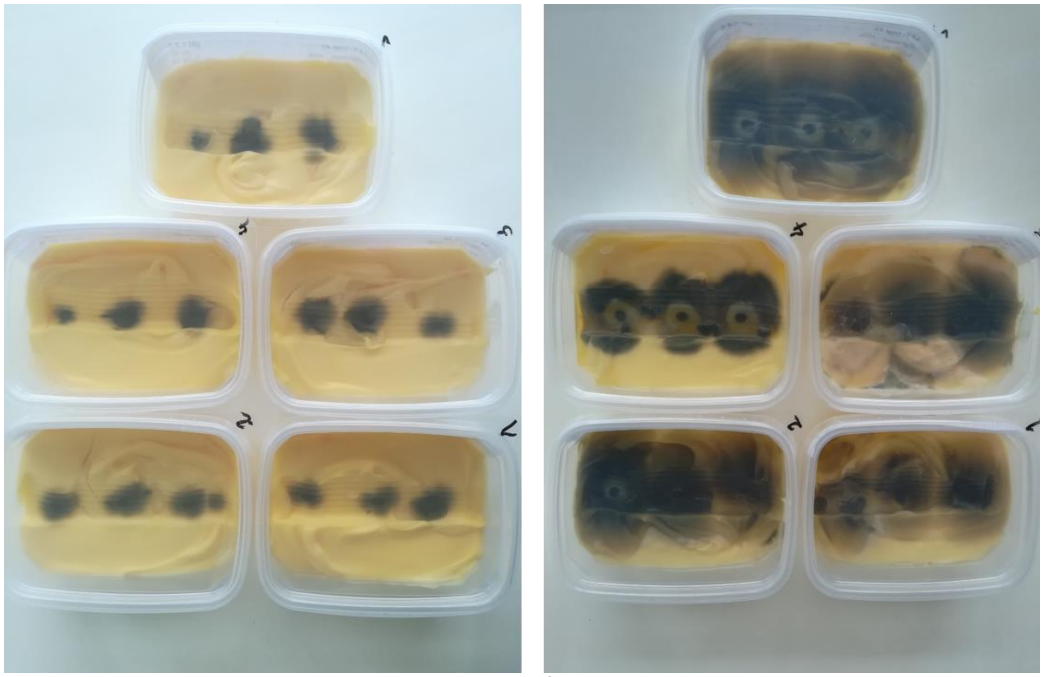


Figure 6.6. *Cl. ramotenellum* growth on 40% fat W│O emulsions at pH 3.5 (left) and pH 4.5 (right) at 22 °C at day 182.

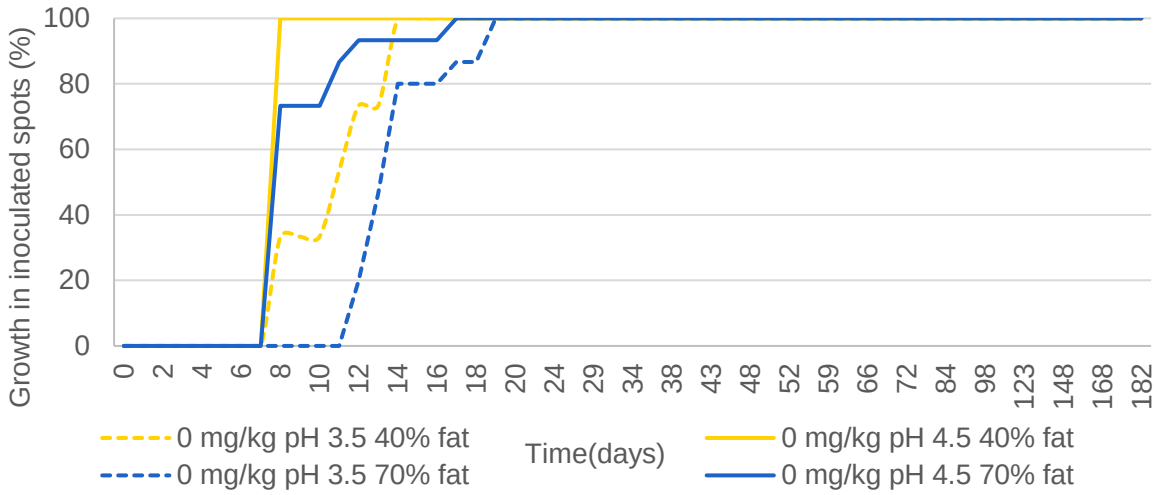


Figure 6.7. Growth (% inoculated spots) of *Cl. ramotenellum* in 40 and 70% fat W│O emulsions at pH 3.5 and 4.5 during 182 days at 22 °C.

6.3.3.3. Growth of *P. commune* in 40 and 70% fat W│O emulsions at pH 3.5 and 4.5

As seen in the discussion for the previous two molds, a lower $(HA)_{aq,eq}$ inhibited mold growth in W│O emulsions than in YEG media. *P. commune* didn't grow in 70% fat W│O emulsions, irrespective of the pH and temperature (Figure 6.8 and 6.9). Interestingly, growth started at the same time in 40% fat W│O emulsions at pH 3.5 and 4.5 at 7 °C, while at 22 °C growth started earlier at pH 4.5 than at 3.5, as observed for the previous two molds.

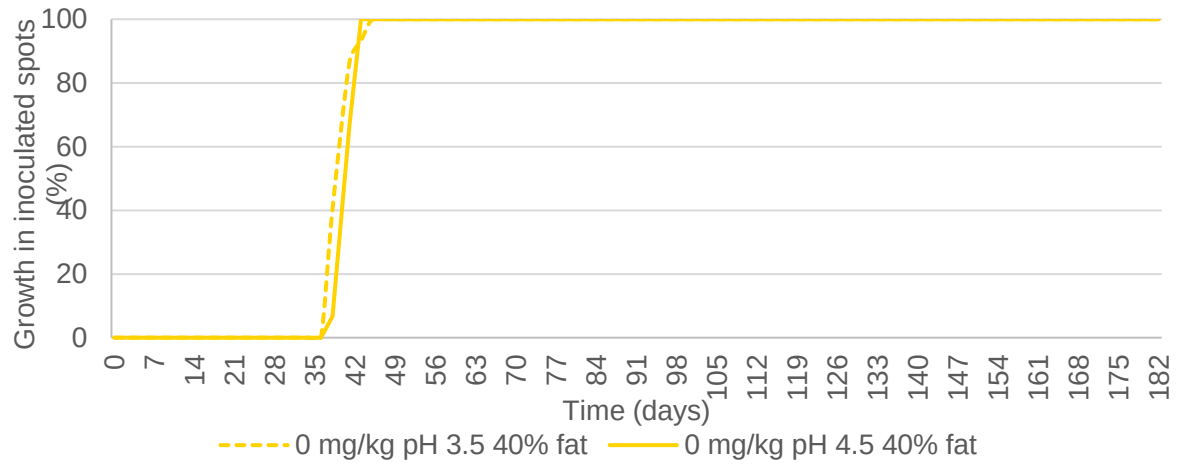


Figure 6.8. Growth (% inoculated spots) of *P. commune* in 40 and 70% fat W│O emulsions at pH 3.5 and 4.5 during 182 days at 7 °C.

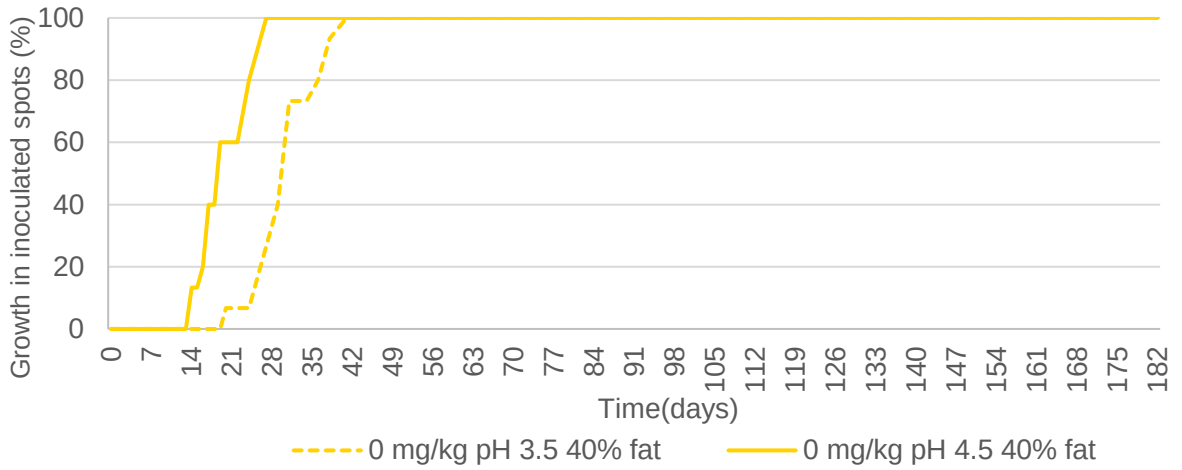


Figure 6.9. Growth (% inoculated spots) of *P. commune* in 40 and 70% fat W│O emulsions at pH 3.5 and 4.5 during 182 days at 22 °C.

It is important to notice the order of growth observed in the inoculated W│O emulsions. Generally, the molds first started growing in 40% fat W│O emulsions at pH 4.5, at both 7 °C and 22 °C, other than *P. commune* at 7 °C .

The structure of a W│O emulsion is dictated by the properties of the continuous (lipid) and discontinuous (aqueous) phase. A 40% fat W│O emulsion will have a coarser water droplet size distribution than a 70% fat W│O emulsion, due to the larger volume of the aqueous phase. van Zijl and Klapwijk (2000) stated that the decrease in fat content in many W│O emulsions can have consequences for the microbiological stability of the aforementioned products. Delamarre and Batt (1999) found that the size of the aqueous phase droplets and the inability of microorganisms to move between droplets reduce the ability of W│O emulsions to support

microbial growth,. However, ter Steeg et al. (2001) claimed that molds are able to grow through the fat matrix, but no experimental data was provided to confirm this claim.

In conclusion, complete inhibition of growth of all three tested molds in 40 and 70% fat W|O emulsions was achieved by applying between 171 and 219 mg/kg $(HA)_{aq,eq}$, depending on the pH, r and f_{tot} of the W|O emulsion. $(HA)_{aq,eq}$ MICs in W|O emulsions were generally lower than those elucidated in the YEG media. As for the unpreserved W|O emulsions, all of the tested molds started growing earlier in 40% fat W|O emulsions than 70% fat W|O emulsions, while *P. commune* didn't grow in 70% fat W|O emulsions. Growth mostly occurred first in W|O emulsions at pH 4.5 than at 3.5. As expected, all the molds grew faster at 22 °C than at 7 °C. Of the three tested molds, *Cl. ramotenellum* grew most comfortably, in both 40 and 70% fat W|O emulsions at pH 3.5 and 4.5. *P. commune* grew only in 40% fat W|O emulsions and not in 70% fat W|O emulsions while *A. niger* grew only at 22 °C and not at 7 °C.

CONCLUSION

The preservation of W|O emulsions relies on manipulating a complex set of intrinsic and extrinsic factors. Most microbiological problems in W|O emulsions can be attributed to mold spoilage. The same type of molds spoil various types of W|O emulsions, such as those belonging to *Aspergillus* spp., *Cladosporium* spp. and *Penicillium* spp.

Data obtained using spectrophotometric measurements have been utilized with the aim of calculating the minimum inhibitory concentration (MIC) of a compound under specific sets of environmental conditions. One of the most important aspects of developing microbiological growth screening methods is ensuring that the predictions are applicable to real situations, a process termed validation. The majority of fast screening methods have been developed in liquid laboratory culture media. Due to the fact that food W|O emulsions are structured products, it was decided that it was more appropriate to follow mycological growth in semi-solid media. It was confirmed that the spectrophotometric measurement method in semi-solid media proposed by Medina et al. (2012) was successful in screening the susceptibility of various emulsion spoiling molds towards sorbic acid in YEG semi-solid media.

The results of the spectrophotometric measurements in YEG semi-solid media showed that, for all three molds, the $((HA)_{aq,eq} + (A^-)_{aq,eq})$ MICs increased as pH increased. When comparing the three molds used in our study, the most sensitive towards sorbic acid, low temperature and low pH was *A. niger*. On the other hand, *P. commune* was the most resistant to the above

mentioned conditions, and couldn't be inhibited at pH 5.5 by the maximum sorbic acid concentration allowed in emulsions, 2000 mg/kg. The obtained MICs were validated in industrially produced W│O emulsions andno mold growth was observed in neither 40 nor 70% fat W│O emulsions with sorbic acid during 182 days.

During W│O emulsion formulation, it is necessary to take into account the loss of sorbic acid in the lipid phase. Thus, to ensure a full inhibitory effect of sorbic acid, the amount of the preservative to be added to the total mass of the system needs to be higher in W|O emulsions rather than only aqueous matrices. This was calculated over the proposed preservative distribution model (Chapter 2) and necessary $(HA)_{tot,eq}$ were calculated to obtain a $(HA)_{aq,eq}$ that corresponded to the MICs elucidated in YEG media. Generally, less $(HA)_{aq,eq}$ was necessary to inhibit mold growth in W│O emulsions than in YEG semi-solid media. This could be a consequence of the emulsion structure limiting fungal outgrowth but also the fact that microbiological growth media allows for the most comfortable outgrowth which in turn increases the quantity of preservatives necessary for inhibition. These findings emphasize the importance of validation of microbiological preservative sensitivity studies in culture media on real food matrices.

To our best knowledge, this study is the first study of its kind where sorbic acid MIC values for emulsion spoiling molds were calculated over a proposed preservative distribution model and validated in semi-solid media and in industrially produced W│O emulsions.

GENERAL CONCLUSION AND PERSPECTIVES

Oil and fat based foods represent a large proportion of the energy intake in human diets around the world. Food W|O emulsions, such as butter, margarine and fat spreads, are an important source of fat in human diet. Butter and margarine are an indispensable part of daily diets and vital ingredients used in the making of confectionary and bakery products. Transparency Market Research (2019) reports that the global butter and margarine market has been thriving as a consequence of improved purchasing power of consumers across the globe, but also as a consequence of the introduction of a wide range of innovative products and increasing consumption of food items such as pastries, cookies, cakes, and other confectionary items.

Good microbiological stability of food is obtained by applying techniques like pasteurization or by stabilization of the product by its chemical composition and physical nature (intrinsic stability) or MAP packaging and low temperature (extrinsic factors). While these factors can be successfully applied and controlled during the time that the product is still in the production plant, some preservation strategies, like adequate storage temperature, aren't always ensured on distribution or consumer level. Nevertheless, it is in the best interest of the producer to apply the most effective preservation strategies *in-house* in order to minimize the chance of product safety and quality issues arising during consumer use.

Foods, like any organic matter, will deteriorate in time due to biological, chemical and physical factors. In order to prevent or retard inherent decomposition, food preservatives are added to food during product formulation. Concerns about food safety and quality have driven the food processing industry to invest in research regarding preservative behavior and preservative alternatives. Product research and development are essential for food companies to remain viable, but the processes are costly and failure rates are high (Bower, 2013).

In this study, the influence of sorbic acid, the most common used weak organic acid preservative in emulsions, on the fungal stability of W|O food emulsions was investigated.

Elucidating the behavior of sorbic acid in biphasic systems such as W|O emulsions was an intricate task. First, because of the inherently complex matrix of W|O emulsions, second because of the very limited information in literature regarding this topic and third because of the lack experimental validation of theoretical hypotheses postulated in the available literature. This, on the other hand, made the topic relevant and scientifically challenging. As W|O emulsions are complex biphasic systems with many components in both the aqueous and lipid

phase, phenomena in this study were examined by simplifying the food matrix into its principal components before experimental and real food validation.

In order to avoid unnecessary and costly experimental work, many scientific domains recognize the value in modelling and computer simulations as tools for product and process understanding, design, optimization and control. The purpose of a mathematical model is to capture relevant features of a complex object or process, based on existing theoretical understanding of the occurring phenomena and available information. Compared to chemical engineering, where modelling is now part of virtually any scientific and technical development, food science and technology follows a similar trend, with a considerable (~20 years) delay. One of the main reasons for this delay is the increased complexity of food systems, including physical, chemical and biological phenomena on a wide range of time and space scales (Perrot et al., 2011). Modelling of phenomena in food allows food scientists to understand processes in food more clearly, but also to control and make predictions about them. Thus, modeling aids the effort for greater and more consistent food quality and safety. A modelling approach was also followed in this work, by describing mathematically the behavior of sorbic acid in W|O emulsions in order to be able to predict its behavior.

The theoretical model describing the distribution of sorbic acid in W|O emulsions was developed and validated on model W|O systems (Chapter 2). The predictions took into account the varying pH of the aqueous phase, mass fraction of the lipid phase and presence of solid fat in the system. The developed model is a valuable contribution in the quantification efforts of inhibitory concentrations of weak organic acid preservatives in complex biphasic systems. The lipid phase in these systems influences partitioning of small lipophilic molecules, like weak organic acid preservatives, effectively reducing their inhibitory concentrations in the aqueous phase. This, in turn, is seen as a loss of the preservative effect of the antimicrobial compound on microorganisms present in the aqueous phase. Although assumptions of this behavior were already given by Wilson et al. (2001) and an alternative undissociated sorbic acid calculation postulated in the publication of ter Steeg (2001), a more precise development and experimental validation of the occurring phenomena was laid out in this study. The assumptions of Wilson et al. (2001) mentioned above didn't take into account the SFC of the W|O emulsions, but combined the oil and fat present in the system into one factor. In our study, it was assumed that the solid fat in the system was inert, i.e. doesn't allow for the absorption of solutes and took that hypothesis into account during model development. Calculations were also performed with the assumption that solid fat wasn't inert and that it allowed for the absorption of sorbic acid, but in that case the differences between calculated

and measured $(HA)_{aq,eq}$ values were very high, reaching up to 177.5% difference and systematically increasing with the increase of SFC in the system.

To our best knowledge, this work is unique because sorbic acid (weak organic acid) partitioning behavior was experimentally validated in the common pH range of W|O emulsions and the influence of varying oil content on $(HA)_{aq,eq}$ was elucidated and experimentally confirmed. The proof of the assumption of SFC influencing weak organic acid partitioning is an important contribution. The theoretical $(HA)_{aq,eq}$ predictions differed from the measured $(HA)_{aq,eq}$ values at low pH and high SFC in the matrix, more specifically, the measured $(HA)_{aq,aq}$ values were always higher than the theoretical values, rendering this model as a fail-safe model. This could be beneficial news for the emulsion producer, as it implies that the presence of solid fat in the W|O matrix hinders the partitioning process of weak organic acid preservatives from the aqueous to the lipid phase. The reasons for this phenomenon could be attributed to the reduced amount of liquid oil for the sorbic acid to partition into or a change of the water-oil K_p of sorbic acid once solid fat is added. The latter is an interesting hypothesis to investigate further as this would mean that the K_p of a W|O emulsion could vary with the SFC of the emulsion. It is interesting to mention that Teles dos Santos et al. (2014) developed a computational tool that aids in predicting SFC in fat-based products which would allow combining both models. Integration of several computational models during product formulation could reduce the need for costly experimental work.

Initial experiments regarding the influence of emulsification on sorbic acid distribution were also conducted and the experimental data were compared with the predictions of the model. Even though there was more sorbic acid recovered in the lipid phase of the emulsified system than the non-emulsified system, the experimental setup was too narrow to make a definite conclusion. Nevertheless, first steps towards making a laboratory-scale stabile liquid food W|O emulsion which can afterwards be separated were made and this should aid in future research on this topic.

It is worth mentioning that the preservative distribution model developed in this study was recently used for predicting the inhibitory concentration of thymol, a phenol found in thyme oil, applied to a bread product (Debonne et al., 2019). At present there are strong trends amongst consumers and retailers towards preservative-free products. This has put pressure on producers to devise various strategies investigating alternatives to conventional preservatives. One strategy commonly used is the application of essential oils. It is thought that, due to their extensive antimicrobial properties, many essential oils could be used for microbial control, preservation of food safety and quality and prolongation of food shelf life. Essential oils are

“Generally Recognized as Safe” and due to their natural origin are better accepted by consumers than “synthetic” preservation agents. These strategies are also being tested in the emulsion making industry, but haven’t proven more effective than conventional preservatives. Also, the use of these compounds is limited by organoleptic properties and legislation restrictions. The sorbic acid distribution model could be used to predict the inhibitory concentration of other small lipophilic compounds found in essential oils and applied to (food) matrices containing oil and water compounds. The scope of the model application could also be oriented not only to W│O emulsions, but also to O│W emulsions, bakery and confectionary products which contain water-soluble and lipid-soluble components, vegetable spreads with oil added such as hummus or “ajvar” (traditional pepper spread made on the Balkan peninsula), or even topical emulsified drugs and skin and hair cosmetics. In the future, this knowledge could be also expanded by investigating the synergy or antagonism of other aqueous phase solutes and sorbic acid. For example, it would be interesting to investigate the influence of salt or protein, common constituents of the aqueous phase of food emulsions, on the partitioning behavior and efficiency of sorbic acid.

The main purpose of developing the sorbic acid distribution model was to be able to predict aqueous sorbic acid concentrations in W│O emulsions. The knowledge of these concentrations is important because microorganisms primarily inhabit the aqueous phase of W│O emulsions., which was also confirmed in this study. It follows that it is only the aqueous sorbic acid concentrations that will influence microbial growth in the aqueous phase. It has been extensively documented that it is the undissociated aqueous sorbic acid that will exhibit the primary antimicrobial effect, however this form is also the only one able to partition into the lipid phase. If the lipid phase is comprised of only liquid oil, the preservative effect on microbial growth will be lower than if it is comprised of liquid oil and solid fat, as was confirmed in this study. The increased inhibitory efficiency of sorbic acid when solid fat is present was confirmed by the complete inhibition of *C. guilliermondii* in model W|O+F systems at pH 4.5 compared to its comfortable growth in model W|O systems, where sorbic acid partitioned to the liquid oil phase thus diminishing its preservative effect). This also implies that solid fat in the system could help prolong exposure time of microorganisms to sorbic acid.

The work in this study was primarily oriented on investigating the influence of sorbic acid on the growth of fungi (yeasts and molds) in W│O emulsions which are common spoilage agents in such products and where molds are the primary agents of visible spoilage. Deepening the knowledge on the growth behavior of spoilage fungi in W│O emulsions ultimately helps aid reduction of food waste. As mentioned before, predictive modelling was used in describing the generation time and lag phase duration of the yeast *C. guilliermondii* in model W│O systems.

Modelling fungal growth in W|O emulsions can be done using mechanistic or empirical models. Mechanistic or semi-mechanistic models include parameters from equations of applicable known theories (growth kinetics, substrate utilization rates etc.) with the goal of describing the phenomena during growth. Mechanistic models are more routinely used in biotechnology and chemical engineering where there is a need to optimize microbial growth and metabolite production, rather than to control it, as is the case in most food applications. Empirical predictive models describe the experimental conditions of the study (e.g. effect of the physical and chemical components of the food on fungal proliferation). Since these models are descriptions of experimental conditions, it is advised to be careful when using these models to make predictions outside the limits of the original experiments.

While modelling yeast growth can be performed in a similar manner as bacterial growth, modelling mold growth in a food product is a more complicated task. The complex structure of foods makes the measurement and prediction of filamentous fungal growth within a product difficult. Although most of the mold spoilage of foods occurs at the surface because of the aerobic nature of the microorganism and O_2 restriction within the product, it has been stated that fungal hyphae can penetrate the three-dimensional matrix of foods. However, experimental confirmation of this statement in W|O emulsions is lacking. Quantification of mold growth is most simply and directly performed by measurement of hyphal extension rate, usually reported as radial growth rate in µm/h. Gibson and Hocking (1997) suggested that it is relevant to develop more mechanistic models that describe hyphal extension rate. An experimental step to visualize mold growth behavior in W|O emulsions might be in modern microscopy visualization solutions with contrast staining of the structured matrix and inoculated molds. A need of fungal growth models incorporating a broad temperature range has been elucidated, because most models are derived from data at 25, 30 and 37 °C. Ideally, a broader range of temperatures is required to produce a reliable model explaining mold growth dependence on temperature, but this might not be an easy experimental task due to the long experimental times necessary to provide growth data at lower temperatures. Collaborative efforts by several groups of researchers, using standardized methodology, need to be stablished to produce sufficiently large experimental databases to enable growth predictions that are similar to the situation encountered in foods.

The fungal stability of W|O emulsions depends to a large degree on the physicochemical aspects of the matrix. Fungal growth is confined to the aqueous phase and is determined by DSD, nutrients and pH of the aqueous phase, as well as the presence of preservatives. The rate at which these parameters affect product quality is influenced by storage temperature. A satisfactory prediction of the keeping quality of W|O emulsions requires at least two types of

information. First, it is necessary to establish the type of fungi present in the product and second, to determine whether intrinsic (i.e. structural and compositional) conditions and/or extrinsic (storage temperature, atmosphere) conditions of the product favor the growth of spoilage fungi.

Existing empirical knowledge of fungal stability of W|O emulsions was obtained by making flexible model W|O systems allowing for data acquisition and investigating fungal spoilage of various types of products taking into account matrix compartmentalization and antimicrobial preservation factors. Most of the knowledge describing this behavior has been brought forward by Verrips and Zaalberg (1980) and ter Steeg et al. (2001). These investigations were carried out to see if the degree of compartmentalization in the products could be used to safeguard the microbiological stability of the products just like intrinsic parameters such as the presence of preservatives, pH or low a_w. The first parameter mentioned that might have influenced fungal growth in W|O emulsions was DSD and D_{43} of the W|O emulsion (measured by NMR). Investigations in our study also confirm the influence of DSD of W|O emulsions on fungal stability in recombined butter inoculated with *C. guilliermondii.* Namely, DSD and D_{43} changes during time were followed and fungal stability of recombined butter was enhanced when DSD of the aqueous phase was inclined towards small droplets. This means that the more narrow the DSD, and consequently the smaller the D_{43}, the less space there was in droplets of the aqueous phase for *C. guilliermondii* to proliferate. This was the case in full-fat recombined butter, whose structure, reflected in the narrow DSD and a relatively small D_{43}, didn't allow for fungal growth. Fungal proliferation was also inhibited or retarded when keeping temperature was low. The effect of temperature on fungal growth is well known and documented, with higher temperatures favoring faster spoilage. It is important to note that during NMR measurements it was observed that nomenclature of the instrument output wasn't uniform amongst the used NMR machines and software used for DSD calculations, making interpretation of data more complicated. Unifying DSD nomenclature throughout NMR machinery and DSD software could contribute to faster interpretation and comparison of emulsion DSD data. Another recommendation would be to routinely combine microbiological analyses with NMR measurements of $D_{43,}$ DSD and SFC. Information about these parameters provides information about the physical structure of the product, which is an important overall quality aspect.

The second parameter influencing fungal growth in W|O emulsions mentioned in the study perfomed by ter Steeg et al. (2001) is the volume fraction of droplets with a diameter > D_{min} (the minimal diameter for supporting fungal germination and outgrowth) which is a strong indicator of the fungal vulnerability of an emulsion. The probability of fungal outgrowth could

be proportional to the volume fraction of droplets with a diameter larger than D_{min}. It was reported that the size of a water droplet has to exceed an estimated minimal value (D_{min} of 6.5 µm gave the best fit in their approach) to allow fungal spore germination and formation and extension of a germ tube/mycelium to penetrate a second droplet. When experimentally confirming the hypotheses presented above, the authors stated that the D_{43} of W|O emulsions allowing challenge testing should not be too fine ($D_{43} < \pm 6.0$ µm), because this fine structure will be microbiologically stabile and no effects of preservatives might be observed. However, the structure should also not be too coarse, because the droplets of coarse emulsions may coalesce and the DSD may not remain stabile during experiments. A drawback of this method was the high inoculum numbers that were used for model validation. In the publication it was unclear what is the true minimum droplet radius necessary for a droplet to be occupied by a microorganism in case of low contamination levels. However, this value will depend on the contaminating microorganism in question and the amount of nutrients in the droplet. It was also concluded that there is no direct method available to determine exactly the distribution of microorganisms over droplets in W|O emulsions with a low degree of contamination. This could perhaps be achieved by modern contrast microscopy techniques, but the approach would be laborious since large numbers of droplets would have to be evaluated to get a statistically reliable answer. Nevertheless, they observed that the DSD of emulsions with a D_{43} < 15 µm remained stabile over 6 months.

It is important to note that in several literature sources it was stated that molds are able to grow through the fat matrix. However, the information was first given by Macy in 1929 and experimental validation of this claim in food emulsions is lacking. It would be interesting to inoculate food emulsions with a mold strain tagged with fluorescent proteins and follow its growth during time with, confocal microscopy. Also, although Brocklehurst et al. (2003) claimed that microbial proliferation in W|O food emulsions is accompanied by coalescence of aqueous phase droplets, this was only true in liquid/liquid emulsions. Confirming or disproving this information in W|O food emulsions with solid fat would be an important step in clarifying the consequences of fungal proliferation in discrete aqueous phase droplets.

The third parameter influencing fungal growth mentioned by ter Steeg et al. (2001) that remains unexamined is the influence of the growth-limiting nutrient in the water phase on fungal proliferation. In order to conduct these experiments, one would first need to identify which is the growth-limiting nutrient in the common aqueous phases of W|O emulsions. Also, it should be determined if the choice of the growth-limiting nutrient is species dependent. The rate at which a microorganism utilizes the growth-limiting nutrient is called the yield coefficient, K. This is a species dependent parameter and will also contribute to fungal growth in the aqueous

phase of an emulsion. When estimating fungal growth in a W│O emulsion, one should use the yield coefficient of the most efficient substrate metabolizing microorganism (i.e. the most mass of cells produced per least mass of growth-limiting nutrient consumed). When determining this value it is crucial to determine if the K obtained from experiments in liquid (or semi-solid) culture media is comparable to the K in structured W|O emulsions. Another factor to take into account is the conversion of nutrients into inhibitory products. Although the three factors governing fungal stability mentioned by ter Steeg et al. (2001) weren't closely examined in this study, they are worth examining in further research with the aim of minimizing spoilage of food W|O emulsions.

An important contribution of this study to the existing knowledge on the role of sorbic acid in the fungal stability of W│O emulsions is the validation of the sorbic acid distribution model on real food products. Namely, fungal studies are often performed in laboratory scale conditions, and it is rare to be able to confirm the lab-scale findings on industrial scale. Gibson and Hocking (1997) mentioned that modelling of fungal growth hasn't developed as rapidly as bacterial modelling because of the difficulties of acquiring sufficient satisfactory data and the lack of support from food manufacturers. This is understandable due to the large chance of cross-contamination of food products with fungal spores when conducting mycological tests in-house. However, collaboration between food manufacturers and research institutions specialized to study mycological growth could prove to be a step forward towards combining manufacturing and research know-how with the goal of ensuring safe and wholesome food for the consumer. This study is also a step forward in that direction.

The real-food model validation was performed by first screening emulsion spoiling molds for sorbic acid sensitivity in semi-solid microbiological culture media, made to mimic structured food products. The sorbic acid MICs elucidated in semi-solid media were then validated in industrially produced W|O emulsions by using the proposed sorbic acid distribution model and taking into account the pH, mass ratio of lipid phase and SFC of the products to calculate the necessary $(HA)_{tot,eq}$ in order to achieve the $(HA)_{aq,eq.}$ that had shown to be inhibitory in semi-solid media.

Less sorbic acid was needed to inhibit mold growth in industrially produced W|O emulsions than in semi-solid microbiological culture media. This could be due to the difrerence in nutrient composition of semi-solid media and the aqueous phase of the W|O emulsion but also due to the contribution of emulsion structure on sorbic acid and its influence on fungal stability. These findings elucidate the importance of validation of results in microbiological culture media on real food matrices. Brocklehurst (2003) claimed that a validated model should be consistently

“fail-safe”, that is, predictions should fail on the side of safety (e.g. predicted growth rate and lag times should be faster and shorter, respectively, than experimental values). The lower sorbic acid concentrations inhibiting mold growth in industrially produced W|O emulsions than in semi-solid microbiological culture media follow the above mentioned recommendation for successful model validation.

During experimental design of the validation experiments in semi-solid media and industrially produced emulsions, several questions regarding the setup and analysis methodology of fungal stability of W|O emulsions had risen.

First, the method of incubation for the screening test needed to be decided upon. Namely, classical plating of fungi is a laborious and material-intensive process. Thus, it was decided to use a method that can provide a larger set of data in a shorter time than classical plating, more specific - OD measurements of fungal growth in semi-solid media. Fungal growth quantification approaches for bacteria and yeasts using OD measurements in microtitre plates have been suggested by the end of the last century, and the question was if the growth of molds in liquid culture medium was comparable to that in a structured food product. This approach has proven to be suitable to screen the susceptibility of emulsion spoiling molds towards sorbic acid in semi-solid nutritive media. It is important to note that OD change over time may not always be directly related to viable counts, i.e. predictions might not directly be related to the growth that might occur in a product. Nevertheless, this approach allows assessment of the effects of different levels of inhibitors, as seen in this study.

Another technique that might provide large amounts of data quickly is flow cytometry. This technique has the advantage of providing information about the microorganisms on a cell-by-cell basis, but it can also be suitable for measurements of cell size, interaction, aggregation or shape using non-labelled cells by means of analyzing their light scattering characteristics. A publication has recently been issued reviewing flow cytometry applications on filamentous fungi (Bleichrodt and Read, 2019). The studies mentioned in this review demonstrated that flow cytometry and fluorescence-activated cell sorting (FACS) are powerful tools to study fungal germination rate, to identify, compare and separate different types and sizes of spores or to analyze germination rates of different types of spores produced by the same fungus.

Challenge testing in microbiology is a process of deliberate inoculation of a food product in order to examine the growth potential of the tested microorganism in the given conditions. Challenge testing of fungi in emulsion producing facilities often isn’t a viable option due to the

high potential of microbiological cross-contamination of real products by fungal spores and the difficulties that would emerge to resolve such a hazard.

One issue that was faced while performing challenge tests investigating the fungal stability of W│O emulsions was the method of inoculation of the product. The choice of method should depend on the purpose of the experiment. If one wants to determine the growth potential of a fungi in the W│O matrix, the distribution of fungi in the water droplets of an emulsion or the influence of fungal growth on D_{43}, the obvious method of choice would be inoculation of the aqueous phase during initial mixing (as seen in Chapter 5 of this study) or post mixing the inoculum with the finished product (as seen in ter Steeg et al., 2001). In this manner, the fungal cells would be distributed throughout the W│O emulsion matrix. This method would also be useful when challenge tests with pathogenic and spoilage bacteria in W│O emulsions are performed. Using this method, the question arises if the mixing or stirring technique reduces the inoculum size, but further experiments in our laboratory demonstrated no effect of emulsification with Ultra Turrax on inoculum size (results not shown in this study).

When the sensitivity of emulsion spoiling molds towards sorbic acid on industrially produced fat spreads was investigated, the inoculation method of choice was surface inoculation because molds are strict aerobes and are primarily responsible for visible spoilage of food products. It is also more representative for surface contamination during filling and packaging, a well-known problem in the sector. Here, also two methods of inoculation could have been applied, dry and wet inoculation. Dry inoculation is applied when airborne contamination by mold spores is investigated (Burgain and Dantigny, 2016) and involves very low numbers of mold spores on the food product (e.g. 1-9 spores per surface). Wet inoculation was the procedure followed in this study and the purpose was to assess the growth potential of the molds in the worst case scenario, i.e. if the product contained free water on the surface or condensation of water on the surface occurred. This method of inoculation might also be relevant when investigating product deterioration on consumer level.

Another parameter to take into account when performing challenge tests on foods is the inoculum level in the matrix. Steels et al. (2000) investigated the influence of inoculum size on the MIC concentrations of sorbic acid necessary to prevent the growth of *Zygosaccharomyces bailli.* They claimed that the MIC increases with the size of the inoculum, where large inocula at high cell density require considerably higher concentrations of inhibitors to prevent growth than do dilute cell suspensions. It was concluded that this effect could be caused by the diversity in the populations of the yeast cells, with higher probability of sorbic acid-resistant cells being present in large inocula. As mentioned for the inoculation method, the choice of

inoculum size also depends on the purpose of the study. In this study, common industrial post-contamination levels were mimicked when testing for fungal growth behaviour in a matrix. But, when testing for the efficiency of a preservative in microbiological media or food, slightly higher inoculum levels were chosen, in order to be certain about the antimicrobial effectiveness of the compound.

To summarize, fungal spoilage of W|O emulsions is an important issue for emulsion producing facilities because it diminishes the quality of the products and contributes to food waste. Considering that sorbic acid is the primary preservative used to prevent fungal spoilage in such products, a theoretical model of sorbic acid distribution in W|O emulsions has been developed and industrially validated, taking into consideration the pH, mass ratio of the lipid phase and SFC of the emulsion. This work is unique because, even though the hypothesis of sorbic acid behavior in W|O emulsions had been postulated before, it is the first where the model assumptions have been experimentally validated and the influence of solid fat content was confirmed. Also, it is unique because the model hypotheses were validated in industrially produced W|O emulsions. The factors leading to fungal spoilage of W|O emulsions are complex and incorporate knowledge of food microbiology, chemistry and technology. Although there are many questions still left to be answered regarding the fungal stability of W|O emulsions, this study is an important step forward in helping to formulate quality products with a long shelf-life.

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ANNEX I - Growth curves showing the effect of sorbic acid on *A. niger, Cl. ramotenellum* and *P. commune* in YEG media at pH 3.5, 4.5 and 5.5 at 7°C and 22 °C. Growth curves at 0 mg/kg sorbic acid are visualized in grey, growth curves at sorbic acid concentrations where growth was still permitted are visualized in **blue**, while growth curves at sorbic acid concentrations that didn't permit growth are visualized in **orange**.

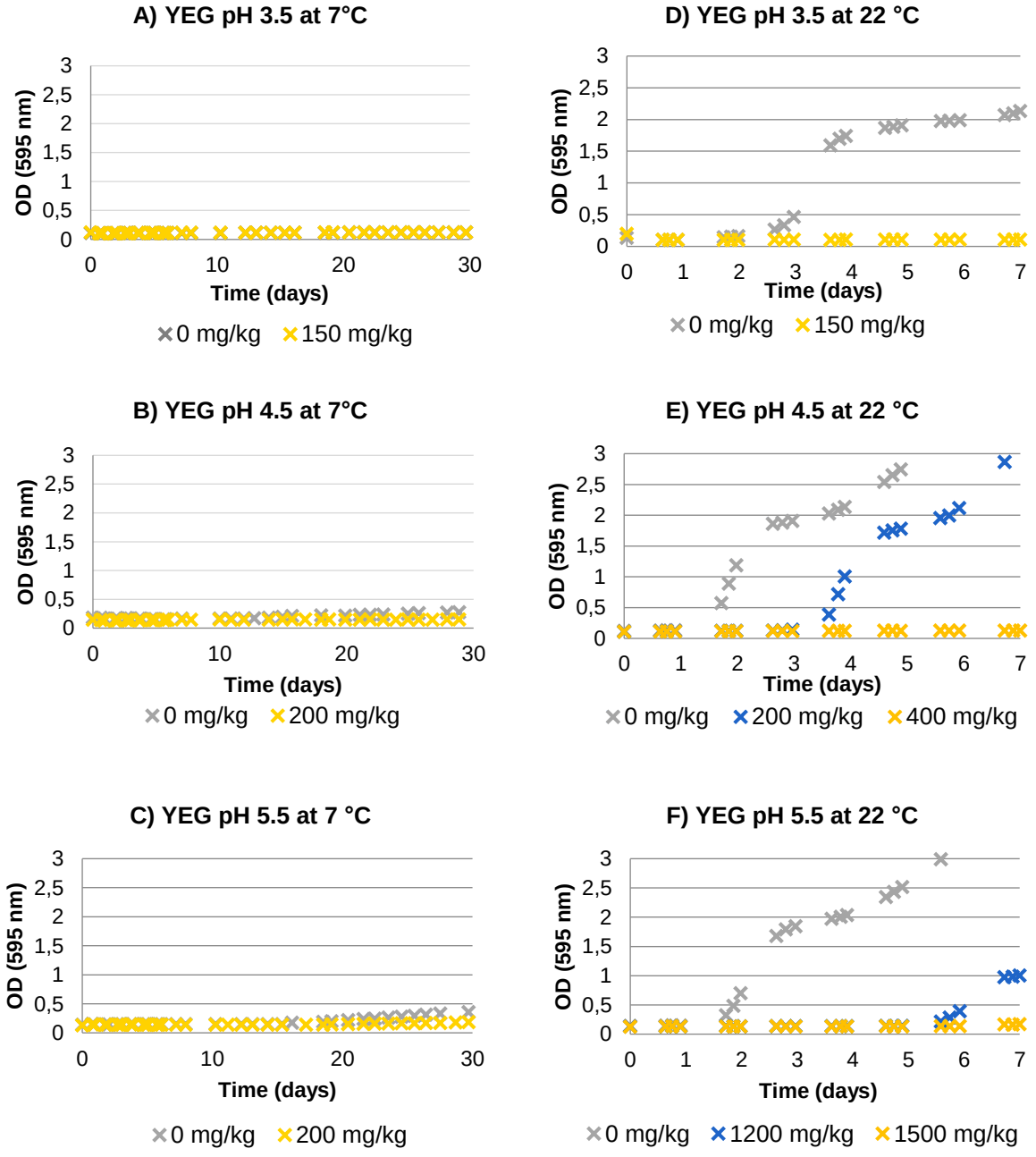

Figure A.1. The effect of sorbic acid on *A.niger* in A) YEG pH 3.5, B) YEG pH 4.5, C) YEG pH 5.5 at 7 °C and D) YEG pH 3.5, E) YEG pH 4.5, F) YEG pH 5.5 at 22 °C. Initial inoculum level was 5×10^2 spores in each well. Each curve is an average of at least 16 replicates.

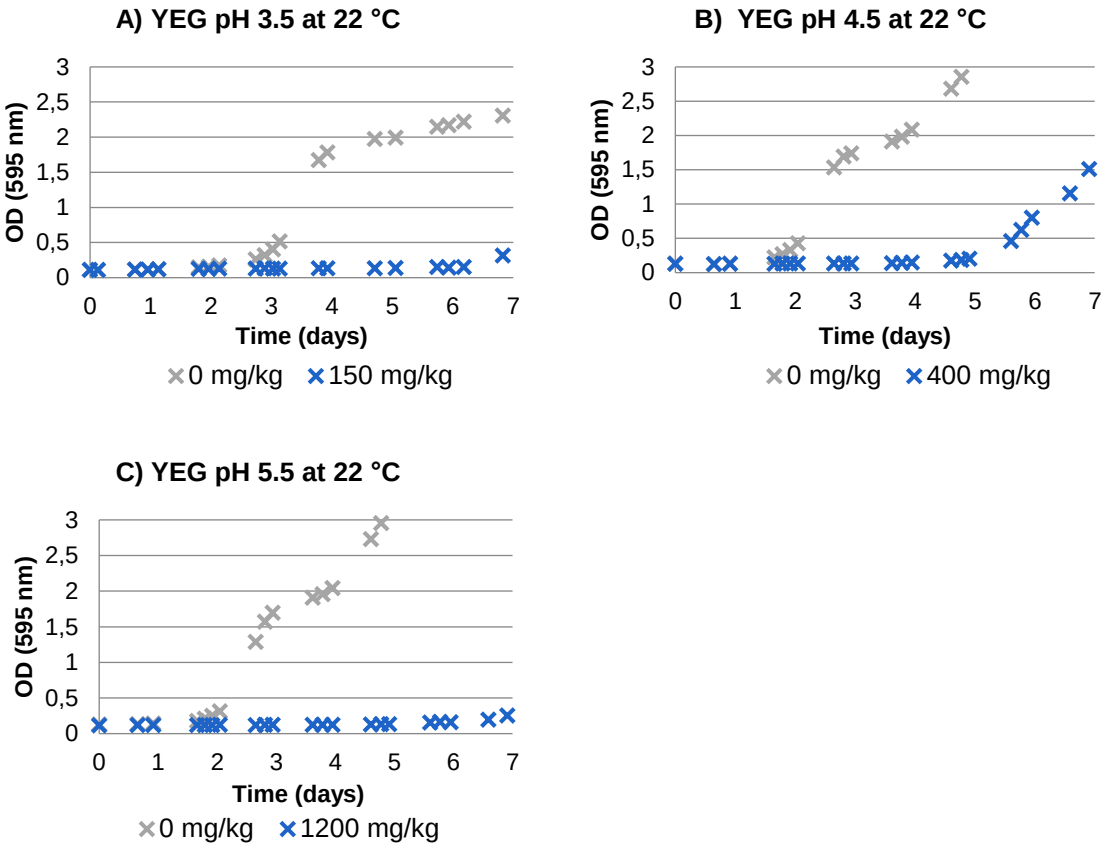

Figure A.2. Follow-up results of the effect of sorbic acid on *A.niger* in A) YEG pH 3.5, B) YEG pH 4.5, C) YEG pH 5.5 at 22 °C. Initial inoculum level was 5×10^2 spores in each well. Each curve is an average of at least 16 replicates.

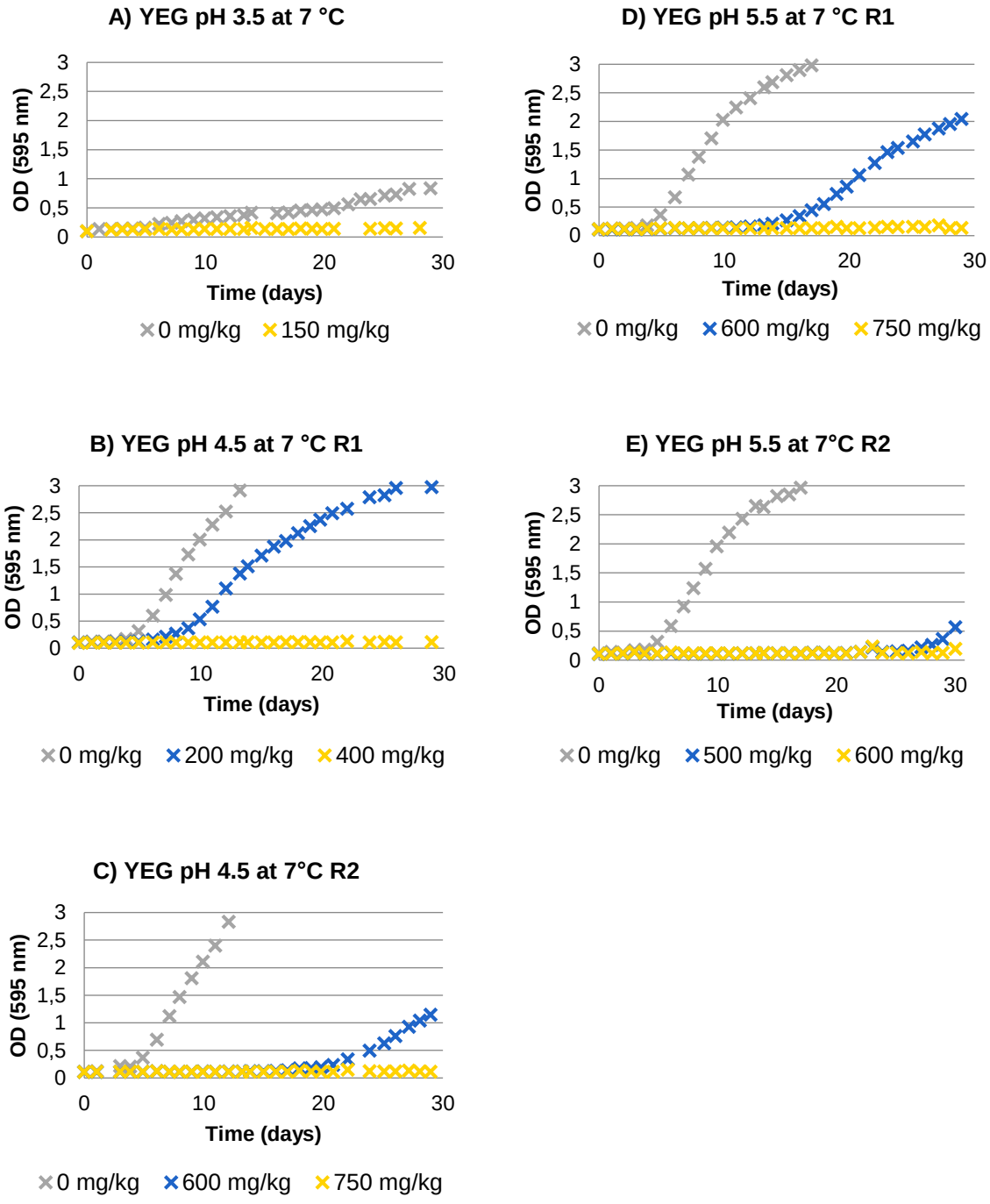

Figure A.3. The effect of sorbic acid on *Cl. ramotenellum* in A) YEG pH 3.5, B) YEG pH 4.5 replicate 1, C) YEG pH 4.5 replicate 2, in D) YEG pH 5.5 replicate 1, E) YEG pH 5.5 replicate 2 at 7 °C. Initial inoculum level was 5×10^2 spores in each well. Each curve is an average of at least 8 replicates.

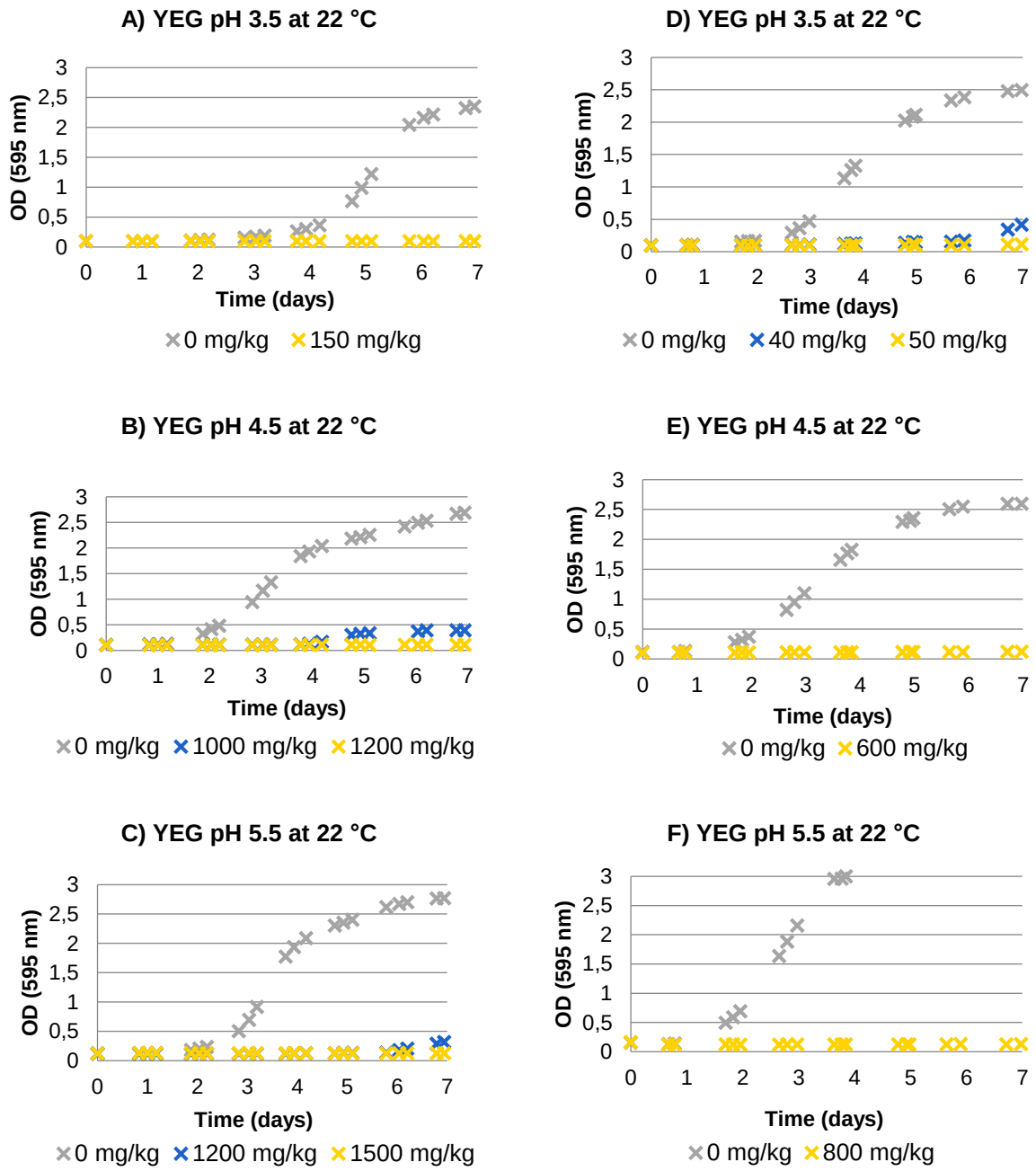

Figure A.4. The effect of sorbic acid on *Cl. ramotenellum* in A) YEG pH 3.5 , B) YEG pH 4.5 and C) YEG pH 5.5 at 22 °C and follow-up results in D) YEG pH 3.5, E) YEG pH 4.5, F) YEG pH 5.5 at 22 °C. Initial inoculum level was 5×10^2 spores in each well. Each curve is an average of at least 8 replicates.

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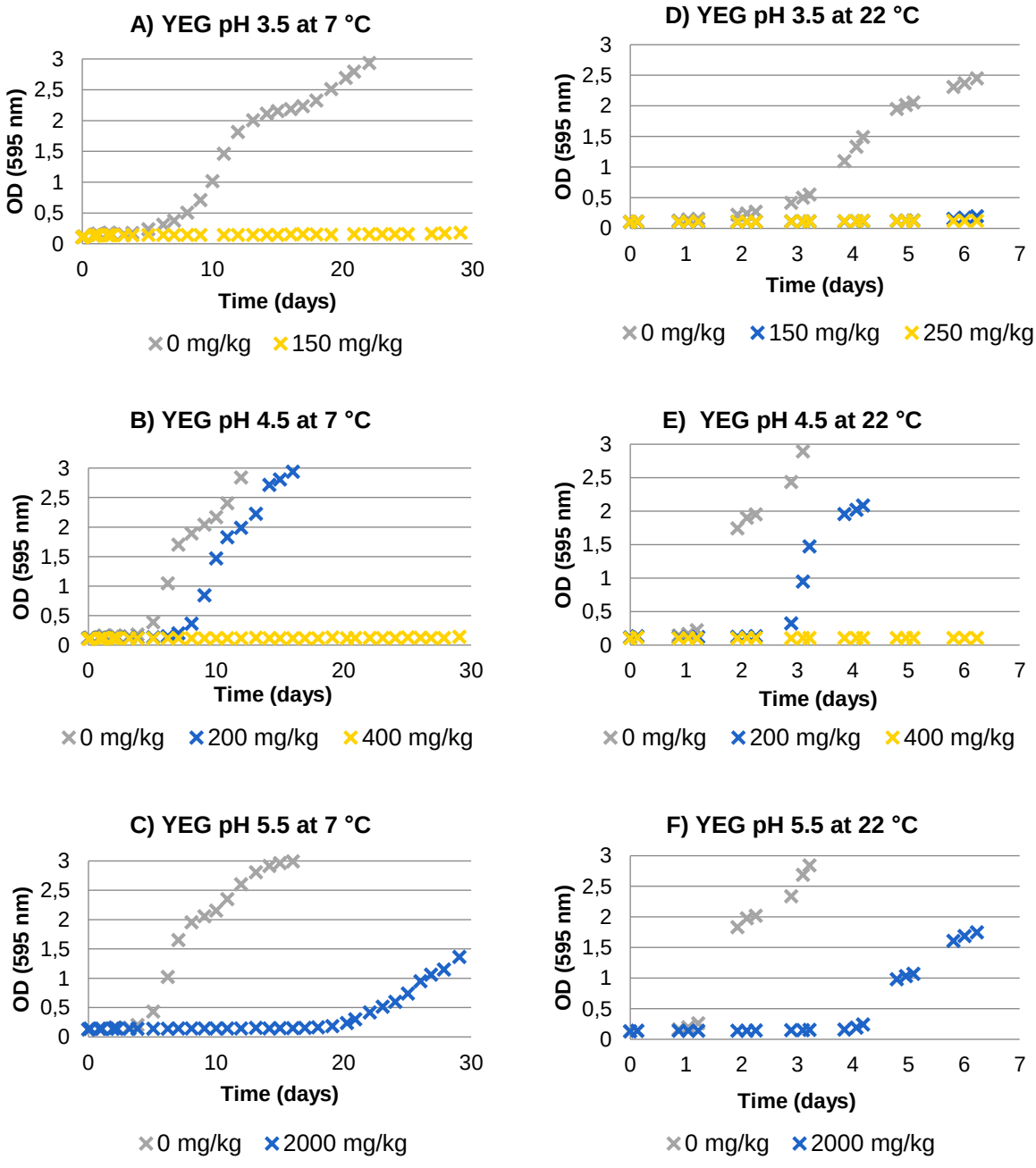

Figure A.5. The effect of sorbic acid on *P. commune* in A) YEG pH 3.5, B) YEG pH 4.5, C) YEG pH 5.5 at 7 °C and D) YEG pH 3.5, E) YEG pH 4.5, F) YEG pH 5.5 at 22 °C. Initial inoculum level was 5×10^2 spores in each well. Each curve is an average of at least 16 replicates in the case of YEG media at 7 °C and 8 replicates in the case of YEG media at 22 °C.

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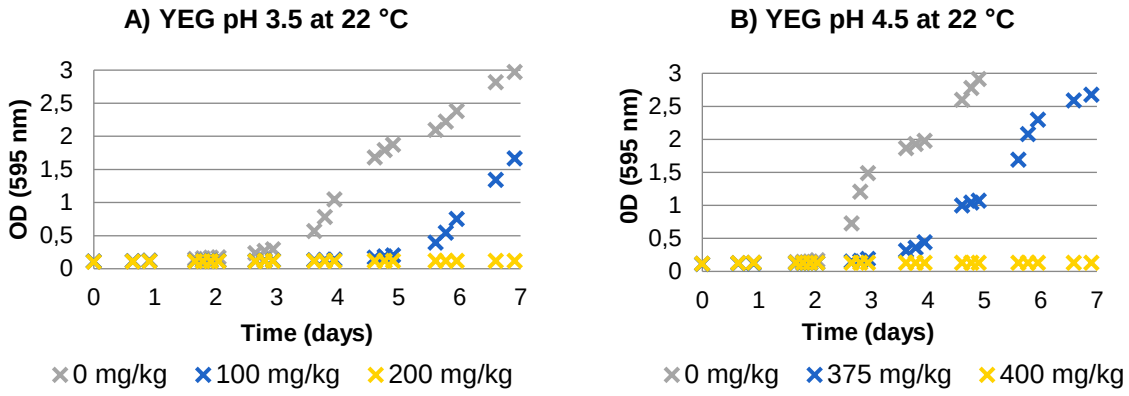

Figure A.6. Follow-up results of the effect of sorbic acid on *P.commune* in A) YEG pH 3.5 and B) YEG pH 5.5 at 22 °C. Initial inoculum level was 5×10^2 spores in each well. Each curve is an average of at least 16 replicates.

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PhD Researcher and Teaching Assistant

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RESEARCH ACTIVITY

Publications in journals with peer review

- **Šoljić, I., Bourdoux, S., Rivière, A., Devlieghere, F.** (2019) Effect of pH, mass fraction of lipid phase and solid fat on sorbic acid distribution in model water|oil+solid fat systems. In preparation. To be *submitted to Food Research International.*
- **Debonne, E., Šoljić, I., Van Bockstaele, F., Eeckhout, M., Vermeulen, A., Devlieghere, F.** (2019) Growth/no-growth models of in-vitro growth of *Penicillium paneum* as a function of thyme essential oil, pH, a_w, temperature. *Food Microbiology.* 83, pp. 9-17.
- **Šoljić, I, Vermeulen, A., Nambooze, C., Rivière, A., Samapundo, S., Devlieghere, F.** (2018) Solid fat influences sorbic acid partitioning and enhances the preservation effect on *C. guilliermondii* in biphasic food model systems. *International Journal of Food Microbiology*. 285, pp. 74-80.

Oral Presentations

- **Šoljić, I., Vermeulen, A., Devlieghere, F.** (2017) Influence of pH and structure on sorbic acid distribution and growth of *C. guilliermondii* in fat-based products. *Predictive Modelling in Food, 10th National conference.*
- **Šoljić, I., Bourdoux, S., Devlieghere, F.** (2017) Influence of pH, fat content and type of fat on the effectivity of sorbic acid in fat based structured food products. *Microbial Spoilers in Food, symposium abstracts.*
- **Šoljić, I., Nambooze, C., Vermeulen, A. Devlieghere, F.** (2016) The influence of liquid oil, solid fat and pH on the inhibitory effect of potassium sorbate on *Candida guilliermondii*. *EFFOST International conference, Abstract.*

Poster presentations

- **Šoljić, I., Abbasian Genaveh, A., Devlieghere, F.** (2017) Advancement of a laboratory scale recombined butter production method used for challenge testing with the spoilage yeast *C. guilliermondii* at refrigeration and room temperature. *IAFP European symposium on food safety, Abstracts.*

Reviews

- **Monono, A., Wiesenborn, D.P., Vargas-Ramirez, J.M., Zhou, R.** (2019) Preserving Juice from Industrial Beets Using Organic Acids. *Transactions of the American Society of Agricultural and Biological Engineers*. 62(1) pp., 177-185.

EDUCATION ACTIVITY

- Responsible for the organization of lectures and practical work of the courses "**Food microbiology and analysis**" and "**Technology of fishery products**".
- Participated in the practical work of the courses "Food analysis" and "International Training Programme Food Safety".
- Tutor of 9 Master and Exchange students during their thesis or internship.

Courses taken include

Data Science Summer School, University of Göttingen (5 ECTS)	July 2017
Introduction to Data Analysis using Excel, Microsoft Corp. [online] (score 95/100)	Feb 2017
The Data Scientist's Toolbox, John Hopkins University [online] (score 100/100)	Aug 2016
Applied Statistics, Penn State Eberly College of Science [online]	May 2016
FLAMES Methodology and Statistics Summer School, University of Gent	Sep 2014

SERVICE ACTIVITY

- Assisterend Academisch Personeel (AAP) representative in the "Doctoral Schools Committee" at Faculty of Bioscience engineering (2016-2017)
- AAP representative in the "Commissie Wetenschappelijke Onderzoek" at Faculty of Bioscience engineering (2014-2016)

Work experiences include:

General Secretary, Global Harmonization Initiative	Nov 2012 – present
Content and PhD contest volunteer, TEDx Ghent	Jun 2015 - present
Freelance food expert, Intertek Group plc.	Sep 2013 – Jun 2017
Technical editor, Food WeB	Sep 2015 – Sep 2016
Laboratory manager, Conex trade d.o.o., Croatia	Sep 2012 – Jan 2013

- Elected in 2012 as **General Secretary of Global Harmonization Initiative** - a network of food scientists volunteering to promote harmonization of global food safety regulations.
- Content team member and main responsible for **TEDx Ghent PhD contest** 2017.
- Employed by **Intertek Group plc**. as a freelance food expert for label validation of food products in Croatian market against European legislation.
- Technical editing and translation of open access online lectures on **"Bridging skill gaps between the food industry in Western Europe and Western Balkans".**
- Laboratory and safety manager at a newly opened fish processing facility in Croatia.
- Recipient of several scholarships for academic excellence including the Scholarship of the University of Zagreb 2011, Biotechnical Foundation in 2010 and Basileus II scholarship for 2009/2010.

EDUCATION

Master of Engineering in food safety management
University of Zagreb, 2012
Bachelor of Nutrition science
University of Zagreb, 2008

SKILLS & INTERESTS

TECHNICAL: Microbial analysis • HPLC • Minitab • Excel VBA • Latex • LANGUAGES: Croatian • English (TOEFL 115/120) • Dutch (B2) • Macedonian • SOCIAL: Communication • Teamwork • Problem solving • OTHER: Entrepreneurship • Piano • Camtasia • Tactical Arbitrage •